THE UNIVERSITY *of* EDINBURGH

CHARACTERISATION, DEVELOPMENT AND APPLICATION OF A CLINICAL MODEL OF THROMBOSIS AND FIBRINOLYSIS

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BSc (Hons) MBChB (Hons) MRCP

Thesis presented for the degree of Doctor of Philosophy at the

The University of Edinburgh

2013

For Niamh

ABSTRACT

The demonstration of antithrombotic efficacy in man is challenging. Most techniques evaluate specific plasma or cellular components under static conditions *in vitro*. In contrast, *in vivo* thrombus initiation and growth occur in whole blood, under conditions of continuous flow and in the presence of vascular injury. An *in vivo* model for use in clinical studies presents significant safety issues and does not currently exist. The Badimon chamber is an *ex vivo* model of thrombosis that is suitable for use in clinical studies and has previously been used to assess novel antithrombotic regimens. Although well-established, previous characterisation studies were performed in a porcine system and using methodology that has since been superceded. In addition, it has a number of disadvantages that limit its broader applicability and has not previously been used to assess fibrinolysis.

Having established The Badimon Chamber within my own institution, I developed the methodology and performed careful validation and characterisation studies with a particular emphasis on reproducibility. These developments allowed more efficient data analysis and the accurate addition of compounds to the extracorporeal circuit, both of which broaden the applicability of the technique. In subsequent studies, using a series of double-blind randomised controlled crossover studies in healthy volunteer cohorts, I utilised the updated methodology to address questions in separate but overlapping areas of cardiovascular medicine.

The dynamic regulation of intravascular thrombus formation by the endogenous fibrinolytic system is central to the pathogenesis of acute atherosclerotic events, particularly within the coronary circulation. Previous work within our institution has

provided novel insights into the role of endogenous fibrinolysis. Despite a growing body of evidence, a key limitation of studies to date is that the effects of acute endogenous t-PA release on *in situ* thrombus formation have not been demonstrated. Is endogenous endothelial t-PA released under agonist stimulation functionally active and able to enhance fibrinolysis of *in situ* thrombus? Firstly, I demonstrated that the addition of exogenous t-PA into the extracorporeal circuit of The Badimon Chamber results in a dose dependent increase in plasma D-dimer associated with a dose dependent reduction in thrombus formation, consistent with enhanced fibrinolysis. Having validated the model, I proceeded to investigate whether freshly released endogenous t-PA would have similar effects to exogenous t-PA. By combining intra-arterial infusion of bradykinin into the human forearm in order to stimulate acute release of endogenous t-PA with an assessment of thrombus formation in the Badimon Chamber, I demonstrated that endogenous t-PA released acutely from the human vascular endothelium enhances fibrinolysis and limits *in situ* thrombus formation. These data validate the forearm model as a relevant model with which to assess acute fibrinolytic capacity, confirm the functional significance of t-PA released during agonist stimulation and suggest that further studies to explore its therapeutic manipulation are warranted.

I went on to evaluate a promising small molecule PAI-1 inhibitor, PAI-749, using assessments of *ex vivo* thrombosis complimented by extensive *in vitro* studies. Interestingly, in contrast to the promising results seen with this compound in preclinical models, we were unable to demonstrate efficacy in any of the clinical models used, highlighting the potential pitfalls of relying solely on *in vitro* and pre-clinical models during early compound development.

In the final phase of this work, I used the chamber to explore the prothrombotic effects of exposure to air pollution. A plethora of observational data exist to suggest that acute exposure to particulate air pollution can trigger vascular events including myocardial infarction although the underlying mechanisms are only partly understood. Using a unique human exposure facility, we demonstrated that inhalation of diesel exhaust causes platelet activation and enhances thrombus formation. These data provide a plausible mechanism linking exposure to particulate air pollution with acute cardiovascular events including myocardial infarction. Furthermore, in a separate study we were able to demonstrate that reducing the particulate component of the exposure using a commercially available particle trap prevents the detrimental effects on *ex vivo* thrombosis and endothelial function. These data support calls for the application of particle traps to diesel-powered vehicles in order to limit a range of adverse cardiovascular effects that result from exposure to traffic-derived air pollution.

DECLARATION

This thesis represents research undertaken in the Centre for Cardiovascular Sciences, The University of Edinburgh, Royal Infirmary of Edinburgh, and the Department of Respiratory and Allergy Medicine, Umeå University, Sweden during the period 2005 to 2009 whilst working as a clinical research fellow.

The characterisation and fibrinolytic work were supported by a British Heart Foundation Project Grant (PG/04/131/18118) that also supported my salary. Additional funding to investigate the effect of PAI-749 was provided by an award (CVDM-EU-008) from the Translational Medicine Research Collaboration. The diesel exposure studies were supported by funding from a British Heart Foundation Programme Grant (PRG/10/9/28286) and the Swedish Heart Lung Foundation.

I have composed this thesis myself. I performed or personally supervised all the characterisation and fibrinolytic work performed in Edinburgh. The investigation of PAI-749, described in chapter 5 was supported by *in vitro* studies performed in collaboration with colleagues at the Universities of Leeds and Aberdeen. The diesel exposure studies were conducted in Umeå, Sweden through a successful ongoing collaboration with colleagues at The University of Umeå. I was personally involved in the design of all studies, the performance of exposure and perfusion studies, vascular assessments, data analysis and production of all manuscripts presented in this thesis. In keeping with the nature of collaborative research, assistance with some of the studies in Sweden was gratefully received from my colleagues Dr Stefan Barath and Dr Magnus Lundback at Umeå University. Assistance was also provided in the technical supervision of the exposures and laboratory assays as acknowledged.

Chapters 3, 4, 5, 6, and 7 have been published in peer-reviewed journals and appropriate copyright permission sought for inclusion of the printed journal manuscripts.

This thesis has not been submitted in any previous applications for a degree and all sources of information have been acknowledged.

All studies were undertaken strictly in accordance with the regulations of and under permission from the Lothian Research Ethics Committee, Edinburgh and The Regional Ethics Board of Umeå, and with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study.

Andrew Lucking March 2013

ACKNOWLEDGEMENTS

This work was performed under the supervision of Professor David Newby (Professor of Cardiology and Consultant Cardiologist) and Dr Nicholas Boon (Honorary Reader and Consultant Cardiologist, now retired) at the University of Edinburgh. I am indebted to them for their guidance, support, dedication and tireless enthusiasm for clinical research. Without their vision and supervision, this work would not have been possible.

I would like to acknowledge the British Heart Foundation for supporting these studies through the award of Project (PG/04/131/18118) and Programme Grants (PRG/10/9/28286).

I would like to thank Professor Badimon, Mount Sinai School of Medicine, New York, for agreeing to let me use his technique, for providing the chamber itself and to Dr Urooj Zafar for his assistance during my time in Mount Sinai, New York.

I would like to thank all the staff at the Wellcome Trust Clinical Research Facility (WTRCRF) at the Royal Infirmary of Edinburgh who provided an outstanding and professional environment in which to conduct clinical research. In particular I would like to acknowledge Sharon Cameron and Finny Paterson whose combined professionalism, flexibility and senses of humour ensured that even under the most intense schedules, time spent at the CRF was always a pleasure!

Thanks to Dana Faratian for histology advice and particularly instruction in the use of Ariol.

I have been fortunate during my the period of this research to have the opportunity to work within an established and expanding collaboration including scientists and clinicians based at The University of Edinburgh as well as at the Universities of Leeds, Aberdeen and Umeå, Sweden and the Dutch National Institute for Public Health and the Environment (RIVM). I have made several trips to Umeå and enjoyed hospitality and friendship as well as the more serious stuff! In addition to my colleagues and collaborators, Magnus Lundback, Stefan Barath, Anders Blomberg and Thomas Sandström, I would like to thank Frida Holmström, Annika Johansson, Margot Johansson and Jamshid Pourazar for all their support - including the ficka! I would also like to acknowledge input from Nuala Booth who has kept me right on some of the more intricate details of the human fibrinolytic system – enjoy your retirement!

I have been delighted and privileged to work amongst a talented bunch of friends and researchers including Ninian Lang, Alan Japp, Ingibjorg Gudmundsdottir, Mark Miller, Rodger Duffin, Anne Burdess and Jeremy Langrish, all of whom have provided a fantastic working environment in which to work in a both a professional and social sense! In particular I have valued the vision and boundless enthusiasm of Nicholas Mills, without whom it is unlikely that the diesel exposure studies would have been completed.

I am indebted to all the volunteers who generously gave up their time to participate in these studies.

Finally my wife, Niamh, and my family. I appreciate the share of the burden you have taken at times during the preparation of this thesis. I love you all and thank you for all your love and support.

ABBREVIATIONS

AAA – abdominal aortic aneurysm

a_2-AP - a_2-antiplasmin

ACE - angiotensin converting enzyme

ACS - acute coronary syndrome

ANOVA - analysis of variance

aPTT - activated partial thromboplastin time

CI - confidence interval

CRP - C-reactive protein

DEP - diesel exhaust particles

ELISA - enzyme-linked immunosorbent assay

FBF - forearm blood flow

IL - interleukin

ICAM - inter-cellular adhesion molecule

MI – myocardial infarction

NO - nitric oxide

NO_2 - nitrogen dioxide

NO_x - nitrogen oxides

PAH - polycyclic aromatic hydrocarbons

PAI - plasminogen activator inhibitor

PE – pulmonary embolus

PM - particulate matter

$PM_{2.5}$ - particulate matter less than 2.5μm in diameter

PM_{10} - particulate matter less than 10μm in diameter

PT - prothrombin time

RME - rapeseed oil methyl ester

sCD40L - soluble CD40 ligand

SEM - standard error of the mean

SMPS - scanning mobility particle sizer

SNP - sodium nitroprusside

SO_2 - sulphur dioxide

TA - tranexamic acid

TAFI - thrombin activatable fibrinolysis inhibitor

TAT - thrombin antithrombin complex

TNF-α - tissue necrosis factor alpha

t-PA - tissue plasminogen activator

u-PA - urokinase

VN - vitronectin

vWF – Von Willebrand factor

WHO - World Health Organisation

CHAPTER 1

INTRODUCTION

Extracts from this chapter have been published in:

Lucking AJ, Newby DE. Pharmacological antithrombotic adjuncts to percutaneous coronary intervention. *Expert Opin Pharmacother*. 2007 Apr;8(6):759-76

Lucking AJ, Chelliah R, Trotman AD, Connolly TM, Feuerstein GZ, Fox KA, Boon NA, Badimon JJ, Newby DE. Characterisation and reproducibility of a human *ex vivo* model of thrombosis. *Thromb Res*. 2010 Nov; 126(5):431-5.

1.1 OVERVIEW

The work described within this thesis was broadly performed in three phases.

The first phase involved establishing the Badimon Chamber, a technique for assessing *ex vivo* thrombosis formation, within my own institution followed by validation of the methodology with a particular emphasis on reproducibility. This phase also involved development and adaption of the methodology to broaden its versatility.

Following successful validation and development, the applicability of the technique was explored by using it in combination with other methodologies to address specific questions within two separate but overlapping areas of cardiovascular medicine.

The *Introduction* chapter of the thesis is divided into sections corresponding to these phases. The final sections focus on the aims and specific hypotheses that are explored throughout the remainder of the thesis.

1.2 INTRAVASCULAR THROMBOSIS

Cardiovascular disease is the commonest cause of premature death in the United Kingdom accounting for 30% of deaths among men and 22% among women. Despite the identification of a number of risk factors, the underlying pathophysiological mechanisms remain only partly characterised. However, it is clear that intravascular thrombus formation is central to the pathogenesis of acute atherosclerotic events, particularly within the coronary circulation. Acute coronary syndromes (ACS) are caused by rupture or erosion of an atherosclerotic plaque leading to exposure of plaque contents and constituents of the vessel wall to flowing blood [Badimon*, et al.*, 2002]. Subsequent thrombus formation may ultimately result in total or partial vessel occlusion with resulting clinical sequelae including sudden death and myocardial infarction (MI) [Badimon*, et al.*, 2002].

Thrombus formation is a complex multistep process consisting of two major pathways: the activation and aggregation of platelets; and the activation of soluble components of the coagulation cascade with the resultant formation of an insoluble fibrin clot [Badimon*, et al.*, 2002]. Due to the rheological conditions within the arterial circulation, the critical initiating factor in arterial thrombosis is the aggregation and adhesion of platelets to collagen and von Willebrand factor (vWF). Subsequent thrombin generation serves to further enhance platelet activation and drive the formation of insoluble fibrin that stabilises the growing thrombus (Figure 1.1).

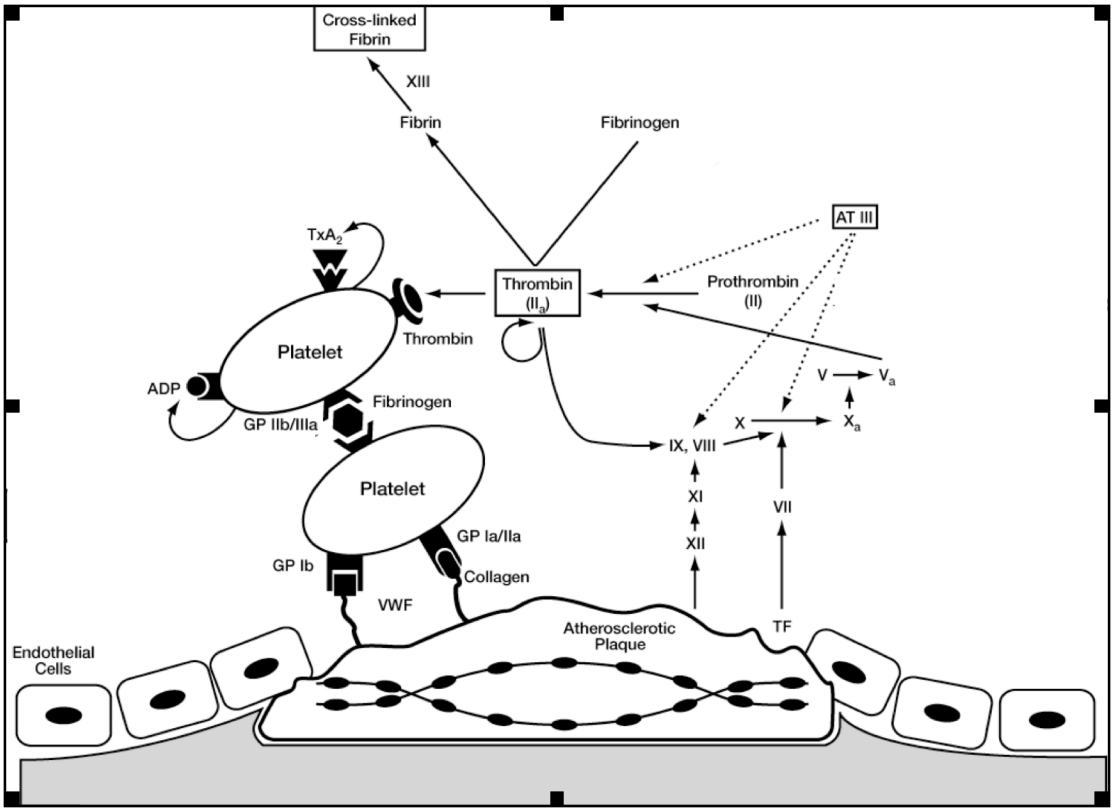

Figure 1.1 *Scheamatic diagram showing the mechanisms responsible for thrombus formation at the site of a ruptured atherosclerotic plaque [Lucking, et al., 2007].*

1.2.1 MEASURING AND ASSESSING THROMBUS FORMATION IN MAN

Given the central role that thrombosis plays in a number of important pathophysiological processes, including ACS, it has been the subject of extensive study. Whilst interrogation of specific components and pathways are valuable, the ability to make an assessment of overall thrombotic potential that results from the net interaction of all relevant factors remains important, both clinically and particularly during the investigation of novel antithrombotic compounds. Whilst several preclinical models exist, species-specific differences ensure that clinical studies remain an essential part of antithrombotic drug development. This has been highlighted in the case of several potential antithrombotic compounds that showed promise *in vitro* and

during preclinical studies but failed to be efficacious in man [Cox, *et al.*, 1992; Hara, *et al.*, 1995].

The demonstration of antithrombotic efficacy in man is challenging. Most techniques involve the evaluation of specific plasma and cellular components in isolation, require the addition of an anticoagulant to limit *in vitro* activation, and are often performed under static conditions. In contrast, *in vivo* thrombus initiation and growth occurs in whole blood, under conditions of continuous flow, and often in the presence of vascular injury. Thus assessment of the efficacy of antiplatelet and anticoagulant agents may not be reflected by static or unidimensional systems employed by *in vitro* assays [Kroll, *et al.*, 1996]. An *in vivo* model for use in clinical studies presents significant safety issues and does not currently exist.

Only a limited number of *ex vivo* techniques have been developed that allow assessment of thrombus formation under controlled conditions [Baumgartner, 1973; Martines, *et al.*, 2004; Sakariassen, *et al.*, 1983]. The Baumgartner chamber was the first popular such technique and consisted of a central rod surrounding by a cylinder [Baumgartner, 1973]. A reverted arterial segment could be mounted on the central rod with the gap between the surface and the cylinder allowing blood to pass over the surface. In addition to being extremely labour intensive, limitations were related to the size and shape of material that could be accommodated within the chamber and difficulties in accurately predicting shear rates at the thrombogenic surface. Subsequently, other *ex vivo* systems were developed that allowed studies using prosthetic surfaces but which were limited by their inability to accommodate biological substrates or study a broad range of flow conditions [Martines, *et al.*, 2004;

Sakariassen*, et al.*, 1983]. All involve an extracorporeal circuit in which blood is perfused over a thrombogenic substrate. The flow rate of blood through the circuit is controlled by a pump and in combination with the lumen of the channel or chamber determines the rheological conditions including the shear stress under which the thrombus forms. A variety of thrombogenic substrates have been used including purified components, extracellular matrix components [Vilahur*, et al.*, 2004], cultured human cells and modified human [Toschi*, et al.*, 1997] and animal blood vessels. Thrombus quantification can be assessed by the use of radiolabelled blood products, immunofluorescence or histomorphometrically.

The Badimon Chamber

The Badimon chamber is an *ex vivo* model of thrombosis that is suitable for use in clinical studies. It provides a powerful and elegant method of assessing *ex vivo* thrombus formation in an extracorporeal flow chamber [Badimon*, et al.*, 1999; Badimon*, et al.*, 1987]. The technique utilises a continuous flow of venous effluent blood from the human forearm that is passed into a series of extracorporeal chambers that sit in a temperature-controlled waterbath.

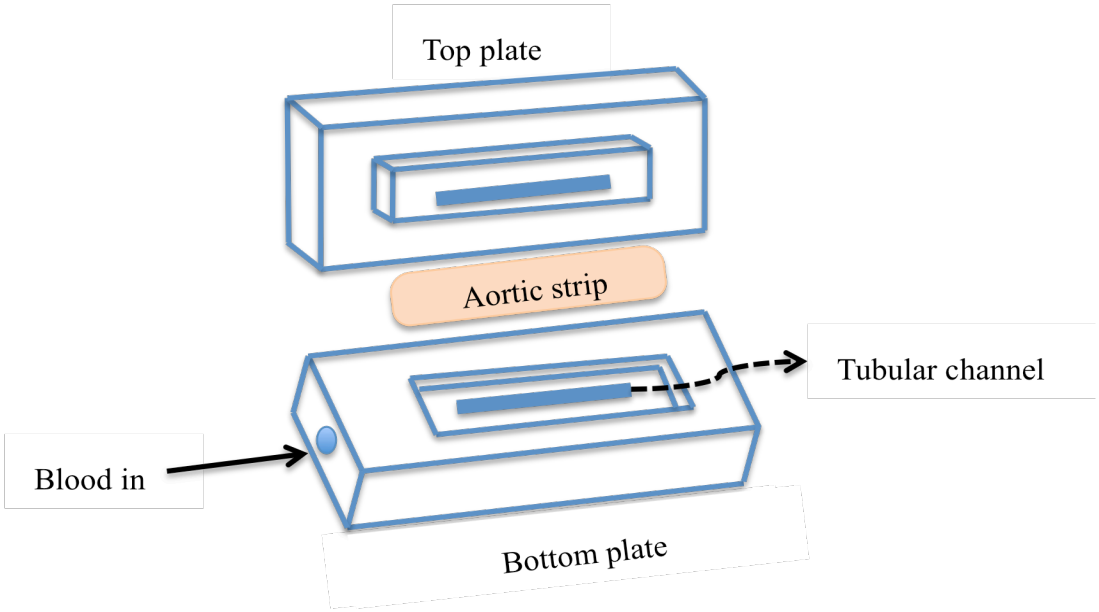

Figure 1.2 *Individual unassembled Badimon Chamber.*

Each perfusion chamber consists of a Plexiglass block through which a cylindrical hole of 1 or 2 mm diameter has been machined in order to mimic the tube-like shape of the vascular system (Figure 1.2). The upper face of the block, parallel to the axis of the cylindrical hole, is milled to create a channel. Biologic or prosthetic substrates can be placed on the channel, with a Plexiglass pressure plate placed on the non-exposed side of the test surface. A ridge on the face of the pressure plate contacts the surface around the periphery of the channel gap and pressure is exerted on the back of the pressure plate by a screw. This compresses the test surface and ensures a sealed system such that blood can be passed through without leakage. For non-compressible surfaces, a gasketing material can also be used. The use of two chambers with different internal diameters was originally decided on by Badimon in order to allow the generation of a broad range of blood velocities and wall shear rates on the substrate with relatively modest changes in the rate of blood flow.

Different biological substrates and biomaterials can be placed within the chamber and

studies using specially prepared porcine vessels, atherosclerotic human vessels [Badimon, *et al.*, 1999; Toschi, *et al.*, 1997], collagen-coated plastic slides [Vilahur, *et al.*, 2004] and prosthetic material [Badimon, *et al.*, 1987; Badimon, *et al.*, 1990] have been completed. The most commonly used substrate consists of specially prepared strips of porcine aorta from which a thin layer of intima has been carefully removed. Flowing blood is thus exposed to the fibromuscular layer of the porcine aorta that comprises of a variety of subendothelial components including collagen (types I and III), proteoglycans, glycosaminoglycans, elastin, and smooth muscle cells. These components are known to be exposed in the ruptured atherosclerotic wall, although probably in different relative proportions with respect to the normal vessel wall. This substrate is highly thrombogenic and induces thrombus formation at any shear rate and perfusion time. In fact, even high shear rates are unable to dislodge significant quantities of platelet thrombi, and deposits continue to increase with exposure time [Badimon, *et al.*, 1986].

Using a blood flow rate of 10 mL/min, the rheological conditions in the first chamber simulate those of patent coronary arteries (channel width 2 mm - low shear rate, approximately 212 s^{-1}), while those in the second and third chambers simulate those of mildly stenosed coronary arteries (channel width 1 mm - high shear rate, approximately 1690 s^{-1}). Whilst blood flow within this system is not pulsatile, rheological conditions otherwise broadly simulate those at the site of intra-coronary atheromatous plaque rupture. Combined with the use of porcine tunica media as a pathophysiologically relevant thrombogenic substrate, the technique serves as a model of deep coronary arterial injury. As such, it is particularly useful for evaluating the efficacy of interventions likely to modulate thrombus formation within the coronary

circulation at a site of vessel injury, including the assessment of novel antithrombotic agents.

Previous studies have utilised the technique to evaluate novel antithrombotic agents or regimens, principally in patients undergoing percutaneous coronary intervention [Lev*, et al.*, 2006; Wahlander*, et al.*, 2006; Zafar*, et al.*, 2007]. It has a number of important advantages over other techniques, particularly the ability to assess thrombus formation on a pathophysiologically relevant substrate and under conditions of continuous flow [Badimon*, et al.*, 1986; Badimon*, et al.*, 1987].

Limitations of the Badimon Chamber Technique

Although the Badimon chamber technique is well-established, previous characterisation studies have been performed in a porcine system and using methodology that has since been superseded [Badimon*, et al.*, 1986; Badimon*, et al.*, 1987]. In addition, there are features of the current technique that limit its broader applicability.

Early animal studies quantified thrombus burden by measuring the quantity of radiolabelled platelets incorporated into the *ex vivo* thrombus [Badimon*, et al.*, 1987]. Given the safety and logistical difficulties of using radiolabelled blood components in human studies, histological methods for assessing thrombus area were developed. Whilst more time-consuming, histological analysis has several important advantages over ^{111}Indium-labelled platelets. By using appropriate conventional staining techniques in combination with immunohistochemistry, total thrombus area and specific components can be quantified [Dangas*, et al.*, 1998; Giesen*, et al.*, 1999]. For

the majority of studies, Masson's trichrome stain is sufficient to quantify both fibrin-rich and platelet-rich thrombus. Histological assessment also allows for the identification of confounding factors such as those caused by gross surface irregularities and flaps that can occur on the denuded aortic strips. Here thrombus formation is driven by tissue disruption rather than the thrombotic state of the subject or the proposed drug intervention. The limitation of histological assessment is that, given the large amount of image data produced, it is extremely time consuming and labour intensive. In previous studies, individual images have been acquired using a microscope-mounted camera before being transferred and analysed using separate image analysis software.

Addition of Compounds to the Extracorporeal Circuit

As discussed, many previous studies using the chamber have been Phase I clinical studies investigating novel antithrombotic agents or regimens and have relied on intravenous or oral administration of the test compound to the subject. Clearly for compounds that require *in vivo* modification, including oral compounds requiring first pass metabolism, exposure of the subject to the test compound is mandated. However, for novel compounds with a prompt mechanism of action and not reliant on *in vivo* modification, there are advantages to being able to assess efficacy without directly exposing the subject to the agent. A significant potential advantage of the Badimon Chamber that has not previously been exploited is the ability to add compounds directly to the extracorporeal circuit of the chamber thus obviating the need to expose human subjects to the agent. This would allow efficacy to be evaluated in a robust and pathophysiologically relevant clinical model using native human blood prior to safety testing. This may allow more timely identification of compounds that show promise

in preclinical models but fail to demonstrate efficacy in man. The ongoing development of such compounds could then be discontinued earlier saving time and money.

However, there are potential limitations to this approach. Firstly, the time available for the compound to mix with flowing blood prior to contacting the chamber is short (~2-3 s). Whilst this may be sufficient for small soluble molecules, it may preclude the testing of larger compounds, particularly where solubility is limited. Although the interaction time could be lengthened by introducing a mixing chamber or reservoir, this would necessitate modulation of blood flow with potential effects on platelet and coagulation factor activation. Secondly, in order to avoid marked dilution of blood as the agent is added, it is necessary add small relatively small volumes of a concentrated solution containing the test agent. For example, to achieve a 1:100 dilution it is necessary to produce a solution 100 times more concentrated than the final desired chamber concentration. Clearly depending on the quantity of compound available and practical issues relating to the production of a concentrated solution, this may not be possible.

1.2.2 SUMMARY

Intravascular thrombosis is central to the pathogenesis of acute atherosclerotic events. The assessment of interventions that may affect thrombosis, including the evaluation of antithrombotic drugs, is reliant on suitable models. Whilst several preclinical models of thrombosis exist, species-specific differences ensure that clinical studies remain an essential part of antithrombotic drug development. The demonstration of antithrombotic efficacy in man is challenging and an *in vivo* model does not currently

exist. The Badimon Chamber provides a means of assessing *ex vivo* thrombus formation in humans, potentially without direct drug exposure, although limitations in the methodology limit its broader application.

1.3 FIBRINOLYSIS

The initiation, modification and resolution of thrombus associated with eroded or unstable coronary plaques is critical in determining the clinical outcome from a complex atherosclerotic plaque. Areas of endothelial denudation and thrombus deposition are a common finding on the surface of atheromatous plaques [Davies, 2000]. They usually remain sub-clinical as endogenous fibrinolysis prevents thrombus propagation [Davies, 2000], although plaque expansion may result as residual thrombus becomes incorporated into the lesion [Mann*, et al.*, 1999]. Indeed, the vast majority of atherosclerotic lesions causing significant luminal stenosis have evidence of previously healed plaque disruption. However, in the presence of an adverse pro-inflammatory state or an imbalance in the fibrinolytic system, microthrombi on the surface of atherosclerotic plaques may propagate acutely, leading to arterial occlusion and tissue infarction [Rosenberg*, et al.*, 1999]. Thus, the initiation, modification and resolution of thrombus associated with eroded and unstable plaques appear to be critically dependent on the efficacy of endogenous fibrinolysis that is itself dependent upon the surrounding endothelium.

In previous studies, novel techniques have provided intriguing insights into the role of endogenous fibrinolysis, endothelial function and intravascular thrombosis in the peripheral and coronary circulations. A greater understanding of these mechanisms and development of a model that allows the assessment of agents with the potential to modulate the endogenous fibrinolytic system is likely to be of scientific interest and has the potential to develop and shape future therapeutic interventions.

1.3.1 THE ENDOGENOUS FIBRINOLYTIC SYSTEM

The endogenous fibrinolytic system protects against intravascular thrombosis and appears to be particularly important in the coronary circulation [Rosenberg*, et al.*, 1999]. Its importance is exemplified by the high rate of spontaneous reperfusion in the infarct-related artery after acute MI, which occurs in around one third of patients within the first 12 hours.

The critical final step of the pathway is the generation of active plasmin from plasminogen, mediated by tissue plasminogen activator (t-PA). Plasmin facilitates clot dissolution through the degradation of fibrin, generating soluble fibrin degradation products including D-dimer (Figure 1.3).

Although the precise mechanism is unclear, t-PA is released from the endothelium through the translocation of a dynamic intracellular storage pool [van den Eijnden-Schrauwen*, et al.*, 1995]. In the absence of fibrin, t-PA is a very weak activator of plasminogen. However, once bound to fibrin, its catalytic activity increases 1000-fold due to conformational changes with the resultant formation of a ternary complex between plasminogen, fibrin and t-PA [Ranby, 1982].

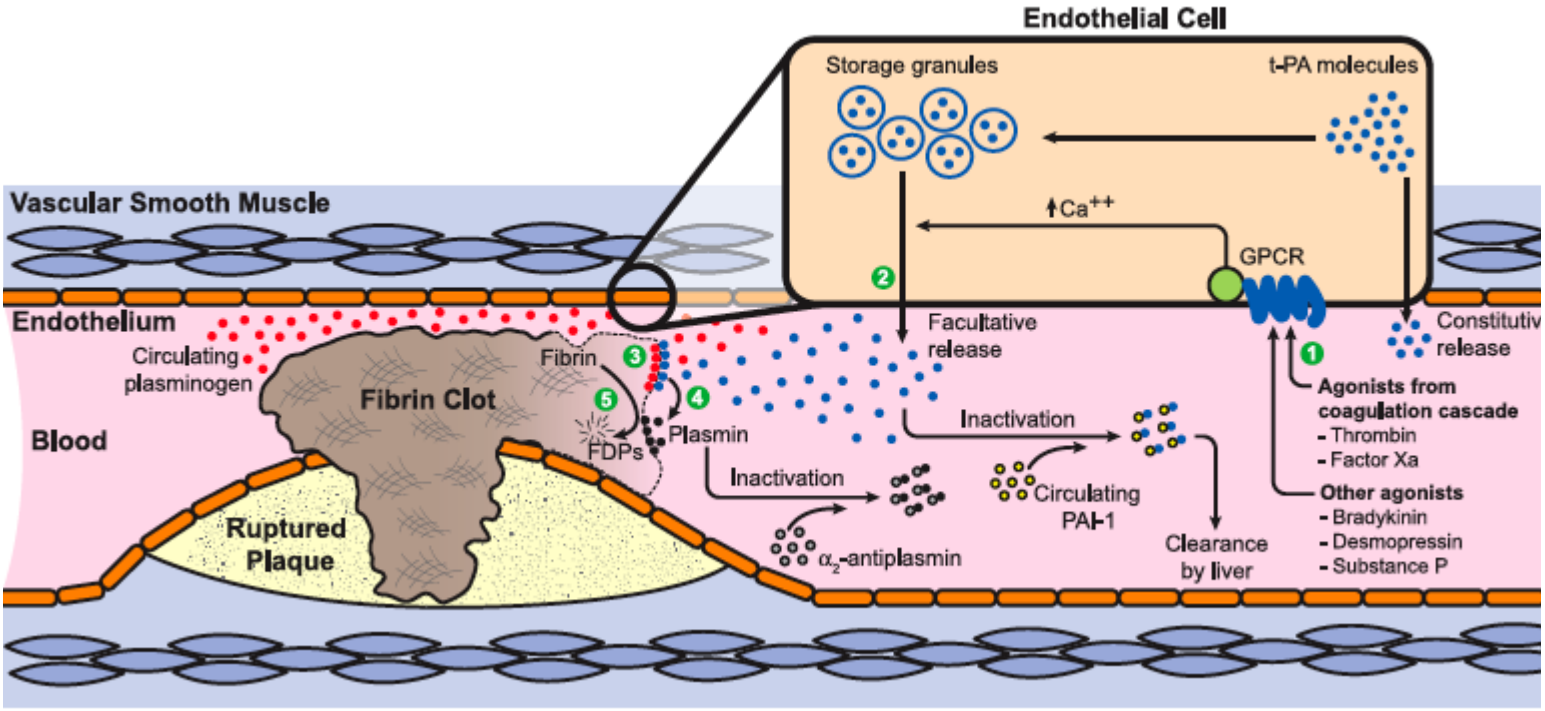

Figure 1.3 *The endothelial fibrinolytic response to luminal thrombus. Agonists generated from the coagulation cascade act on endothelial cell surface G protein-coupled receptors (GPCRs) (1) to stimulate release of tissue plasminogen activator (t-PA) from storage granules, a step that requires an increase in intracellular calcium (Ca^{2+}) concentration (2). Free t-PA acts on thrombus-bound plasminogen (3) to produce plasmin (4) that, in turn, degrades cross-linked fibrin into fibrin degradation products (FDPs) (5), thus dissolving the thrombus. The fibrinolytic process is inhibited by inactivation of t-PA by PAI-1 and other protease inhibitors [Oliver, et al., 2005].*

The rate of plasminogen conversion to plasmin is determined by the release of t-PA from the adjacent endothelium. If endogenous fibrinolysis is to be effective, then the rapid mobilisation of t-PA from the endothelium is essential. The efficacy of the system is determined by the relative balance between the acute local release of t-PA and its subsequent inhibition by a number of serine protease inhibitors, most notably plasminogen activator inhibitor type 1 (PAI-1). PAI-1 normally circulates in substantial molar excess to t-PA and causes its rapid inhibition. In addition, thrombus dissolution is far more effective if t-PA is incorporated during, rather than after, thrombus formation [Brommer, 1984; Fox*, et al.*, 1985]. Many workers have assessed the basal plasma concentrations of t-PA and PAI-1. Whilst potentially informative, these measurements do not reflect the acute fibrinolytic capacity of the vasculature and it is the dynamic release of t-PA from the endothelium that is directly relevant to the pathogenesis of atherothrombosis.

The Dynamic Assessment of Endogenous Fibrinolytic Capacity

In vivo models of assessing acute endogenous t-PA release in the forearm circulation [Newby*, et al.*, 1997] and coronary circulation [Newby*, et al.*, 2001] of man have been developed. In the forearm circulation, using venous occlusion plethysmography combined with bilateral venous blood sampling, local t-PA release has been demonstrated in response to a variety of pharmacological stimuli including thrombin receptor agonism [Gudmundsdottir*, et al.*, 2006], substance P [Newby*, et al.*, 2002; Newby*, et al.*, 1997], tumour necrosis factor α [Chia*, et al.*, 2003] and bradykinin [Labinjoh*, et al.*, 2000].

The Clinical Relevance of Stimulated t-PA Release

Cigarette smoking is strongly associated with atherosclerosis and is also a major risk factor for acute coronary thrombosis [Burke*, et al.*, 1997]. Despite higher basal plasma t-PA antigen concentrations, cigarette smokers have an impaired capacity to release t-PA acutely from the endothelium [Newby*, et al.*, 2001; Newby*, et al.*, 1999]. The increased risk of spontaneous thrombosis seen in smokers may relate to the propagation of thrombus that would otherwise undergo fibrinolysis and remain sub-clinical. Although cigarette smokers have a higher overall mortality from MI than non-smokers [Haheim*, et al.*, 1993], their in-hospital mortality is lower [Barbash*, et al.*, 1993; Mueller*, et al.*, 1992; Zahger*, et al.*, 1995]. This apparent paradox can be explained by the observation that the infarct related artery is more than twice as likely to become patent in current smokers compared to non-smokers following thrombolytic therapy for acute MI [de Chillou*, et al.*, 1996; Gomez*, et al.*, 1993; Zahger*, et al.*, 1995] perhaps because patients with impaired endogenous fibrinolytic capacity are likely to benefit most from thrombolytic therapy compared to those with a preserved ability to release endogenous t-PA who are more likely to have t-PA resistant thrombus.

Bradykinin

As well as being an inflammatory mediator, bradykinin is released during the contact phase of coagulation [Schiffman*, et al.*, 1980]. Patients with unstable angina have increased plasma levels of bradykinin [Hoffmeister*, et al.*, 1995]. When infused into the forearm, bradykinin causes a large dose-dependent release of t-PA that is substantial enough to cause systemic elevations in plasma t-PA concentrations [Labinjoh*, et al.*, 2000]. Thus, the local liberation of bradykinin in acute coronary

syndromes may represent an important negative feedback loop in which bradykinin-induced t-PA release inhibits intravascular thrombus formation. Bradykinin has a very short plasma half-life due to rapid degradation by a number of peptidases, the most important of which is angiotensin-converting enzyme (ACE). Elegant studies in healthy volunteers and patients with chronic heart failure have demonstrated that bradykinin induced t-PA release is markedly increased in the presence of ACE inhibition [Labinjoh*, et al.*, 2001; Witherow*, et al.*, 2002]. Thus, the potentiation of t-PA release by enhancement of endogenous bradykinin activity may account for some of the cardiovascular benefits observed with ACE inhibition.

Endothelial t-PA Release as a Measure of Endothelial Function

The endothelium has a number of important complementary functions, including regulation of vasomotor tone, coagulation, fibrinolysis and inflammation. Most clinical studies have focused on endothelium-dependent vasomotion. Whilst a useful surrogate marker for the role of the endothelium in atherothrombosis [Perticone*, et al.*, 2001], the observation of impaired t-PA release with preservation of vasomotor function in response to endothelium-dependent vasodilators in cigarette smokers [Chowienczyk*, et al.*, 1993] and patients with hypertension [Hrafnkelsdottir*, et al.*, 2004; Hrafnkelsdottir*, et al.*, 1998] highlights both the complexity of vascular biology and suggests that fibrinolytic capacity may be a more sensitive marker of endothelial dysfunction in some circumstances. Indeed, the fibrinolytic capacity of the endothelium predicts future adverse cardiovascular events in patients with coronary disease and may provide additional insights into endothelial function [Robinson*, et al.*, 2007].

1.3.2 ENHANCING FIBRINOLYSIS

Endogenous fibrinolysis plays a critical role in preventing thrombus propagation on the surface of unstable atherosclerotic. In addition, fibrinolytic therapy with recombinant t-PA remains an important treatment in MI and pulmonary embolus. Novel compounds able to accelerate fibrinolysis may therefore enhance the therapeutic efficacy of thrombolytic agents as well as having the potential to prevent adverse cardiovascular events through augmentation of endogenous fibrinolysis.

Work to date on the fibrinolytic system has principally been conducted using plasma-based systems *in vitro* and has allowed the identification and characterisation of the key components. Whilst other plasminogen activators exist, including urokinase-type plasminogen activator, t-PA is the major protagonist of fibrinolysis in plasma. In contrast, several inhibitors of fibrinolysis have been identified including PAI-1, PAI-2, a_2-antiplasmin (a_2-AP) and thrombin activatable fibrinolysis inhibitor (TAFI) [Lijnen*, et al.*, 1995]. Although their function is complementary, they appear to have distinct roles and locations. Others have explored the potential to enhance fibrinolysis by targeting some of the inhibitors of fibrinolysis. The majority of work to date has looked at inhibition of PAI-1 and TAFI.

PAI-1 and PAI-1 Inhibition

PAI-1 is a member of the superfamily of serine proteinase inhibitors and is the primary inhibitor of t-PA *in vivo* [Lijnen, et al., 1995; Pannekoek, et al., 1986]. It is a 379 amino acid glycoprotein produced by several tissues, mainly endothelial cells and vascular smooth muscle cells, but with a contribution from platelets and the liver [Simpson, et al., 1991].

In its active form, PAI-1 limits t-PA activity through the rapid formation of an inactive PAI-1/t-PA complex [Kruithof, et al., 1984; Thorsen, et al., 1988]. The active form of PAI-1 is unstable (half-life 1–2 h at 37 °C) and converts spontaneously into a non-inhibitory latent form [Hekman, et al., 1985] in a process unique among serpins in that it occurs spontaneously at a relatively rapid rate [Levin, et al., 1987; Lindahl, et al., 1989]. A third conformation, the non-inhibitory substrate form, interacts with t-PA resulting in the cleavage and irreversible inactivation of PAI-1 and the regeneration of the proteinase activity [Declerck, et al., 1992; Munch, et al., 1993; Urano, et al., 1992].

In vivo, most plasma PAI-1 is in a complex with vitronectin (VN) [Sigurdardottir, et al., 1990], which stabilises PAI-1 in the active conformation [Lindahl, et al., 1989; Sigurdardottir, et al., 1990; Zhou, et al., 2003]. However, PAI-1 that is stored in the α-vesicles in platelets is not in complex with VN [Robbie, et al., 1996] and is active [Brogren, et al., 2011]. Following platelet activation, this stored PAI-1 is released protecting the primary clot from fibrinolysis [Brogren, et al., 2011; Robbie, et al., 1996] by inhibiting t-PA secreted from nearby endothelium. Even though an inhibitor for PAI-1 that neutralises the activity of both PAI-1 in complex with VN and free

PAI-1 may yield the most effective inhibition, it is still possible that selective inhibition of free PAI-1 could provide therapeutic benefit.

PAI-1 plays a pivotal role in a myriad of physiological processes [Dellas*, et al.*, 2005]. Increased levels of PAI-1 activity have been reported in a broad range of thrombotic disease states including venous thromboembolism, coronary artery disease and acute myocardial infarction [Lijnen, 2005]. In addition, elevated levels of PAI-1 are being increasingly being regarded as central to the pathogenesis of metabolic syndrome. Both glucose and insulin increase PAI-1 synthesis in vascular endothelial and smooth muscle cells [Mertens*, et al.*, 2006] and improved glycaemic control in type 2 diabetes results in a decrease in PAI-1 levels. Some of the benefit derived from statins may relate to their ability to alter the balance of the fibrinolytic pathway, decreasing PAI-1 expression at the same time as enhancing t-PA expression [Bourcier*, et al.*, 2000; Wolfrum*, et al.*, 2003]. PAI-1 has also been linked extravascular disease processes including fibrin deposition and alterations of cell adhesion and migration mediating cancer progression and metastasis [Bajou*, et al.*, 1998]. Inhibition of nitric oxide synthase induces PAI-1 expression that appears to contribute to perivascular fibrosis [Kaikita*, et al.*, 2001].

Given its role in a number of diverse disease states, several strategies to inhibit PAI-1 have been explored. PAI-1 inhibitors include inhibiting peptides, antisense oligonucleotides blocking PAI-1 synthesis [Gils*, et al.*, 2004] and inhibitory monoclonal antibodies [Gils*, et al.*, 2004; van Giezen*, et al.*, 1997]. Finally, a number of small molecule synthetic antagonists have been developed. These inhibit PAI-1 activity by reducing accessibility to the reactive site loop or by inducing

conformational change to a latent- or substrate-like conformation [Elokdah, *et al.*, 2004; Fjellstrom, *et al.*, 2012; Friederich, *et al.*, 1997; Gardell, *et al.*, 2007; Gils, *et al.*, 2002; Gorlatova, *et al.*, 2007].

However, despite considerable interest and several promising preclinical studies, no clinical studies have yet been performed.

TAFI and TAFI Inhibition

TAFI is a zymogen that is activated by plasmin, thrombin or the thrombin–thrombomodulin complex to form an active carboxypeptidase, TAFIa [Bajzar, *et al.*, 1995]. During the partial degradation of fibrin clots by plasmin, new C-terminal lysine residues are generated that further stimulate plasmin formation. TAFIa removes these C-terminal lysines from fibrin, resulting in a decreased rate of plasmin generation and thus downregulation of fibrinolysis [Wang, *et al.*, 1998]. This mode of action is in clear contrast to the working mechanism of PAI-1, which inhibits t-PA by direct interaction.

In humans, a positive correlation has been demonstrated between TAFI levels and the risk for coronary artery disease, venous thrombosis and angina pectoris [Silveira, *et al.*, 2000; van Tilburg, *et al.*, 2000].

Activated TAFI is inhibited (relatively non-specifically) by chelating agents, compounds that interfere with the disulfide bridges, small synthetic compounds and naturally occurring metallocarboxypeptidase inhibitors (e.g. potato tuber carboxypeptidase inhibitor) [Cruden, *et al.*, 2005; Leurs, *et al.*, 2005; Wang, *et al.*,

2007]. In addition inhibition of TAFI has been achieved using inhibitory monoclonal antibodies [Gils*, et al.*, 2005].

As with PAI-1 inhibition, work with novel inhibitors of TAFI has been performed in preclinical models only.

1.3.3 SUMMARY

The outcome of atherosclerotic plaque erosion or rupture is critically dependent on the endogenous fibrinolytic system, particularly in the coronary circulation. In addition, fibrinolytic therapy remains an important treatment for acute pulmonary embolus and myocardial infarction. The fibrinolytic system has been well-characterised and potential targets for therapeutic intervention to prevent and treat cardiovascular diseases identified. *In vitro* and preclinical studies have explored novel agents with the potential to enhance fibrinolysis although no substantive human studies have yet been performed. This is due, in part, to the absence of suitable clinical models in which to evaluate novel compounds.

1.4 INVESTIGATING THE THROMBOTIC EFFECTS OF AIR POLLUTION EXPOSURE

A plethora of observational studies conducted over many years have consistently demonstrated an association between exposure to particulate air pollution and increased cardiovascular morbidity and mortality. Despite the strength of the epidemiological evidence, the pathophysiological mechanisms responsible remain incompletely characterised. Growing evidence exists to suggest that oxidative stress and inflammation are central to both the toxicology of particulate air pollution and the pathogenesis of atherothrombosis. It is possible that nanoparticulate or soluble components may translocate into the circulation, resulting in direct effects on atherosclerotic plaque stability, the vascular endothelium, platelet function and thrombosis. Given the widespread exposure to air pollution, a greater understanding of the mechanisms responsible for its deleterious cardiovascular effects should help to develop and target specific interventions to reduce the impact of environmental air pollution on cardiovascular disease.

1.4.1 AIR POLLUTION AND CARDIOVASCULAR RISK

The association between air pollution and cardiorespiratory morbidity and mortality has been widely established [Anderson*, et al.*, 1996; Dockery*, et al.*, 1993] and there is growing evidence that cardiovascular events are responsible for the majority of excess deaths [Pope, 2000]. The World Health Organisation (WHO) currently estimates three million people die each year of air pollution, representing 5% of the 55 million deaths occurring annually in the world [WHO Statistics] with estimates from the UK government broadly in keeping with this (around 8,000 excess deaths in the UK annually). In the most comprehensive study to date, Miller and colleagues

estimate that long-term exposure to air pollution increases the risk of death from cardiovascular disease by as much as 76% [Miller, *et al.*, 2007].

More recently, several studies have highlighted the risk of acute exposure to air pollution. Short-term increases in air pollution exacerbate existing cardiorespiratory disease leading to an increase in hospital admissions, MI and death [Peters, *et al.*, 2001]. In Edinburgh, a city with relatively low levels of pollution, an increase in particulate matter (PM) of 10 $\mu g/m^3$ was associated with an increase in emergency cardiovascular admissions of 4.8% [Prescott, *et al.*, 1998]. Exposure to air pollution causes myocardial ischaemia during light exercise in patients with coronary heart disease [Mills, *et al.*, 2007] and the triggering of acute MI [Peters, *et al.*, 2001]. Accordingly, the risk of mortality from cardiovascular disease is greater for those living in areas of greater pollution [Dockery, *et al.*, 1993; Pope, *et al.*, 2002].

A recent updated Scientific Statement by the American Heart Association [Brook, *et al.*, 2010] acknowledges a causal relationship between exposure to PM less than 2.5 micrometers in diameter ($PM_{2.5}$) and cardiovascular morbidity and mortality as well as identifying $PM_{2.5}$ as a modifiable factor that contributes to cardiovascular morbidity and mortality.

1.4.2 THE ROLE OF PARTICULATE AIR POLLUTION

Whilst air pollution consists of a complex heterogeneous mixture of gaseous and particulate matter, adverse cardiovascular events are most strongly associated with exposure to fine particulate matter ($PM_{2.5}$) [Dockery, *et al.*, 1993; Miller, *et al.*, 2007]. An important component of $PM_{2.5}$ is nanoparticulate matter generated principally

during the combustion of diesel fuel [Charron, *et al.*, 2005]. These particles, with an aerodynamic diameter ≤100 nm, readily deposit within human alveoli and possess a considerable surface area that likely contributes to their biological toxicity [Donaldson, *et al.*, 2002]. A sizeable observational study by Pope *et al* demonstrated the risk of adverse effects associated with $PM_{2.5}$ was greater than for PM_{10} [Pope *et al.*, 2002] and toxicological studies have also highlighted the increased toxicity of the smaller size fractions [Donaldson, *et al.*, 2000; Donaldson, *et al.*, 2001]. It has been hypothesised that ultrafine particles show more free radical oxidant capacity than non-ultrafine particles suggestive of a greater direct oxidative stress at the particle surface [Li, *et al.*, 1999]. Thus the toxicity of particulate matter appears to relate to the number of particles encountered, their size or surface area, and their chemical composition [MacNee, *et al.*, 2000].

1.4.3 HUMAN EXPOSURE SYSTEMS

Observational studies cannot prove a causative biological effect of air pollution exposure and assessing individual exposure to particles using data from local area monitoring systems is hampered by inaccuracy as personal exposure may be several times higher than corresponding area measurements [Watt, *et al.*, 1995]. Because of the hypothesis that combustion-derived particles are important, studies utilising diesel exhaust remain crucial in determining the health effects of exposure to the combustion-derived component of air pollution [Nightingale, *et al.*, 2000; Rudell, *et al.*, 1994]. The particulate emission from diesel engines is over 100 times higher than that from an equivalent petrol engine with a catalytic converter [Sydbom, *et al.*, 2001]. Over 80% of diesel exhaust particles have a size of <0.1 μm, and these represent a substantial component of the $PM_{2.5}$ fraction in ambient air. Due to their

larger surface area, these submicron particles may carry a large fraction of toxic compounds on their surface [Levsen, 2002]. They are deposited deep within the lung and are only slowly cleared leading to a prolonged time period over which they might exert harmful effects.

1.4.4 POTENTIAL MECHANSIMS

A number of theories have been proposed to explain the association between increased PM and cardiovascular disease, including endothelial dysfunction [Mills*, et al.*, 2007; Mills*, et al.*, 2005; Tornqvist*, et al.*, 2007], myocardial ischemia [Mills*, et al.*, 2007], altered autonomic function [Gold*, et al.*, 2000], systemic inflammation [Tornqvist*, et al.*, 2007] and platelet activation [Nemmar*, et al.*, 2003]. A summary of the potential pathways linking inhaled particles to atherothrombosis and adverse cardiovascular outcomes is presented in Figure 1.4. According to this model, inhaled particles cause pulmonary and systemic inflammation that indirectly impacts on the cardiovascular system. In addition, there is evidence to suggest that smaller particles can translocate into the bloodstream to exert direct effects on endothelial cells and platelets, or may penetrate the vessel wall, promoting atherosclerosis or predisposing to plaque rupture.

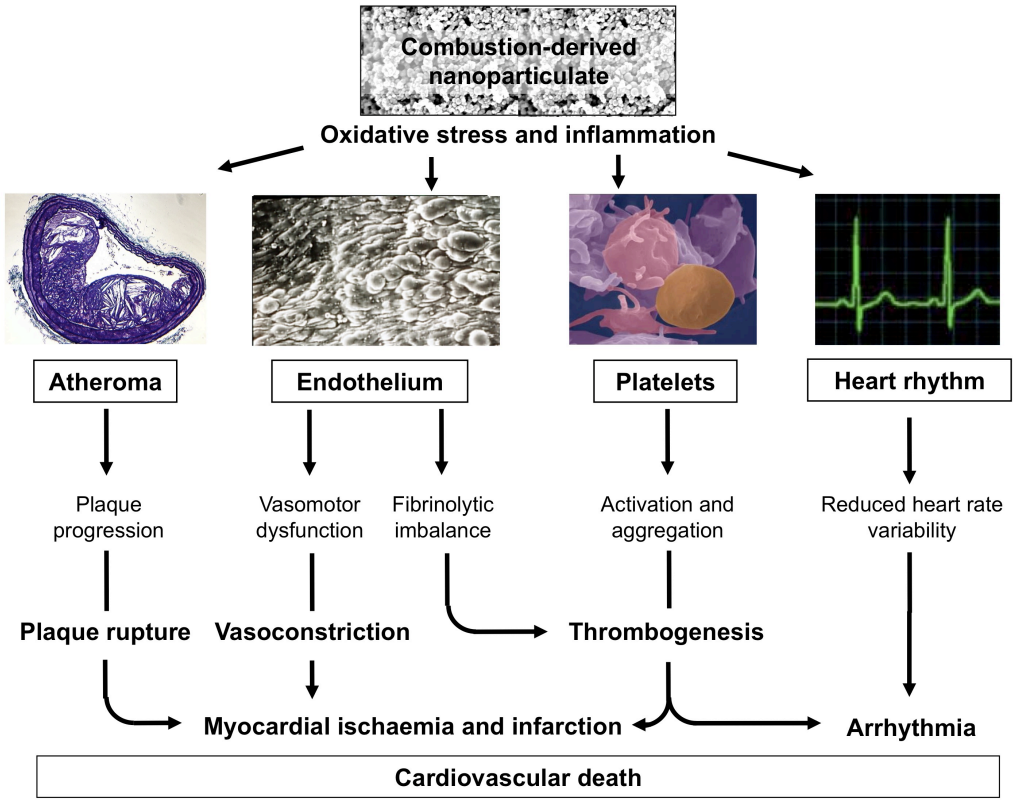

Figure 1.4 *Hypothetical pathways linking inhaled particles to the pathogenesis of atherothrombosis and acute cardiovascular events [figure kindly supplied by Dr NL Mills].*

Endothelial Vasomotor Function and Endogenous Fibrinolysis

In health the vascular endothelium delicately balances regulatory pathways controlling blood flow, coagulation, fibrinolysis and inflammation. It is widely recognised that a variety of risk factors including cigarette smoking can influence vascular tone through endothelium-dependent actions, and there is extensive evidence of abnormal endothelium-dependent vasomotion in patients with atherosclerosis [Celermajer*, et al.*, 1996; Ludmer*, et al.*, 1986; Newby*, et al.*, 1999]. As discussed in the previous section, whilst endothelium-dependent vasomotion is important, it may not be representative of other aspects of endothelial function, such as the regulation of fibrinolysis. Work within our institution has demonstrated impaired endothelial t-PA release in patients with cardiovascular risk factors, including smokers [Newby*, et al.*,

1999]. As combustion products and particulate matter are common to both air pollution and cigarette smoke, we hypothesised that air pollution is likely to have similar detrimental effects on several complementary aspects of vascular function.

Direct Effects of Translocated Particles on the Endothelium

The nanoparticle component of inhaled PM may influence the cardiovascular system through indirect effects mediated by pulmonary inflammation or through the direct action of particles that have become blood-borne. Translocation of inhaled nanoparticles across the alveolar-blood barrier has been demonstrated in animal studies using a range of nanoparticles delivered by inhalation and instillation [Kreyling*, et al.*, 2002; Nemmar*, et al.*, 2002; Nemmar*, et al.*, 2003; Oberdorster*, et al.*, 2002]. Whether inhaled nanoparticulate can readily access the circulation in humans is controversial and continues to be the subject of intense research debate.

Once circulating, nanoparticles may interact with the vascular endothelium, or have direct effects on atherosclerotic plaques causing local oxidative stress and pro-inflammatory effects similar to those found in the lungs. Increased inflammation could destabilise the coronary arterial plaque, resulting in rupture, thrombosis and an acute coronary syndrome. Certainly, injured blood vessels can take up blood borne nanoparticles [Guzman*, et al.*, 1996], a fact exploited by the nanotechnology industry for both diagnostic and therapeutic purposes in cardiovascular medicine. The intra-arterial infusion of carbon black nanoparticles has a detrimental effect on the mouse microcirculation with upregulation of vWF expression and enhanced fibrin deposition on the endothelial surface [Khandoga*, et al.*, 2004]. These prothrombotic effects are in

keeping with toxicological evidence from inhalation studies, which suggest particle exposure may promote thrombogenesis [Nemmar*, et al.*, 2003; Nemmar*, et al.*, 2003].

Direct Effects of Particles on Platelets

Platelets are central to the process of intravascular thrombus formation. Few studies have addressed the direct effects of particles on platelets. In an elegant series of studies by Nemmar [Nemmar*, et al.*, 2003; Nemmar*, et al.*, 2003], *in vivo* thrombosis was induced by endothelial injury using the Rose-Bengal model. In hamsters treated with intra-tracheal diesel particles, both arterial and venous thrombosis was increased in a dose dependent manner. Platelets taken from the instilled animals underwent greater aggregation *in vitro,* although it is unclear whether enhanced *in vivo* thrombus formation was due to inflammation or the direct effects of the particles on platelet function. However, platelets isolated from normal hamsters and treated with diesel exhaust particles showed increased aggregation, suggesting that direct effects were at least plausible.

In a comprehensive study [Radomski*, et al.*, 2005], a range of manufactured nanoparticles including carbon nanotubes, fullerenes, urban PM and carbon nanoparticles were incubated with platelets *in vitro*. Platelet aggregation was assessed and clear differences in the potency of these particles were demonstrated. Fullerenes were without effect, urban dust and multi-walled nanotubes caused modest aggregation, whilst mixed carbon nanoparticles and single walled nanotubes were highly potent activators of platelets upregulating platelet glycoprotein IIb/IIIa receptor expression and enhancing aggregate formation. In a ferric chloride induced rat carotid artery thrombosis model, a similar potency was observed with particles enhancing the

rate of thrombus formation. Unfortunately, there was little characterisation of these particles that might have allowed features such as surface area or chemistry to be linked to the variation in effect on platelet aggregation or thrombus formation.

Effect of Diesel Exhaust on Coagulation

A number of ambient exposure studies have evaluated the association between plasma concentrations of coagulation factors and particulate air pollution with mixed results. Whilst some have demonstrated increased levels of fibrinogen [Ghio*, et al.*, 2000; Mutlu*, et al.*, 2007; Pekkanen*, et al.*, 2000] and vWF [Ruckerl*, et al.*, 2006], other studies measuring the same factors have failed to show any association with particulate exposure [Elder*, et al.*, 2004; Gong*, et al.*, 2003; Ruckerl*, et al.*, 2006]. This apparent disparity may well be explained by variations in study design and perhaps more importantly, the type of exposure investigated. Two previous studies have investigated the effect of controlled diesel exhaust exposure on coagulation factors including fibrinogen, vWF, D-dimer, pro-thrombin fragment 1 and 2, t-PA and PAI-1 in man with neither demonstrating a significant effect [Blomberg*, et al.*, 2005; Carlsten*, et al.*, 2007].

1.4.5 SUMMARY

Exposure to particulate air pollution is strongly associated with cardiovascular morbidity and mortality with multiple plausible mechanistic pathways identified whereby particles may exert adverse effects on the cardiovascular system. Acute exposure to combustion-derived pollutants appears to act as a trigger for myocardial infarction. Given that the majority of such events are due to thrombus formation on the surface of an atheromatous plaque and instillation of diesel exhaust particles

increases thrombus formation in pre-clinical models, it appears reasonable to explore whether these pathways are relevant in man and underpin the association between air pollution and acute cardiovascular events.

1.5 AIMS

The aims of this body of work fell into three principal parts.

1. The first step was to determine whether the Badimon Chamber technique could be established within our centre as a robust method of assessing *ex vivo* thrombus in man. During this process I explored the possibility of whether the process for quantifying thrombus formation could be made more efficient by utilising semi-automated image acquisition and analysis equipment. In addition, I looked at ways to accurately add of compounds to the extracorporeal circuit of the chamber in order to broaden the applicability of the technique.

   Once these initial methodological and validation steps were successfully completed, I used the chamber to address questions with clinical relevance.

2. Recent work within our centre has provided significant insights into the role of endogenous fibrinolysis, endothelial function and intravascular thrombosis. However, the primary end-point in all studies to date has been the quantification of t-PA release. In order to strengthen the clinical relevance of this technique, there was a need to develop a model of *in situ* thrombus formation that would permit the assessment of the relationship between endogenous fibrinolysis and the dynamics of thrombus formation and dissolution. I proposed to resolve this problem by using the Badimon Chamber in combination with the forearm model. Together, they provided an elegant means of stimulating acute t-PA release from the forearm endothelium in order to assess its ability to enhance fibrinolysis and modulate *ex vivo* thrombus formation. In addition,

the technique allows the assessment of modulators of thrombosis and fibrinolysis, permitting the investigation of a broad range of disease processes and mechanisms.

3. Finally, given that preclinical data exist to suggest that thrombosis may be responsible for some of the deleterious effects of acute exposure to air pollution, I explored whether controlled exposure to diesel exhaust could cause platelet activation and/or enhance *ex vivo* thrombus formation and whether these effects could be attenuated by the use of a particle trap.

1.6 HYPOTHESES

The following hypotheses will be addressed:

1. The Badimon Chamber provides a reproducible assessment of *ex vivo* thrombus formation (Chapter 3).
2. Exogenous and endogenous t-PA inhibits *in situ* thrombus formation in the Badimon Chamber by enhancing fibrinolysis (Chapter 4).
3. Inhibition of PAI-1 potentiates dissolution of *in situ* thrombus formation in the Badimon Chamber (Chapter 5).
4. Exposure to diesel exhaust causes *in vivo* platelet activation and enhances *ex vivo* thrombus formation (Chapter 6).
5. The use of a particle trap attenuates the adverse cardiovascular effects of diesel exhaust inhalation (Chapter 7).

CHAPTER 2

MATERIALS AND METHODS

2.1 GENERAL

An overview of the techniques and statistical analysis employed in this thesis is presented below. Details specific to each study can be found in the methods section of subsequent chapters.

2.1.1 SUBJECT RECRUITMENT

Healthy male non-smoking volunteers (aged between 18 and 44 years) were recruited by local advertisement or from volunteer databases held at the Universities of Edinburgh and Umeå. An information sheet was sent to suitable volunteers who fulfilled the inclusion/exclusion criteria outlined below. The general practitioner of each participating subject was informed in writing.

Subjects taking regular medication and those with clinical evidence of atherosclerotic vascular disease, arrhythmia, diabetes mellitus, hypertension (systolic blood pressure >150 mmHg), renal or hepatic failure, asthma, or an inter-current illness likely to be associated with inflammation were excluded. Subjects taking part in the diesel exposure studies had normal lung function, no significant occupational exposure to air pollution and reported no symptoms of respiratory tract infection for at least six weeks prior to or during the study.

2.1.2 ETHICAL CONSIDERATIONS

All studies were undertaken in accordance with the regulations of the appropriate local research ethics committee: Lothian Research Ethics Committee or Umeå Regional Ethical Review Board; and with the Declaration of Helsinki. Written informed consent was obtained from each subject prior to entry into the study.

2.1.3 SUBJECT PREPARATION

Subjects were requested to abstain from alcohol for 24 hours and food, caffeine-containing drinks and tobacco for at least six hours before each study. Studies were conducted in a quiet temperature-controlled room maintained between 22 and 25^{o}C.

2.2 DRUGS

2.2.1 PAI-749

PAI–749, 1-benzyl-3-pentyl-2-[6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-1H-indolemin (MW 502 Da), was synthesised and characterised for chemical identity and purity at Wyeth Research (Collegeville, USA). Aliquots of stock solution (0.5 mg/mL; 1 mM) were prepared in 50% PEG-200/50% sterile water and stored at -80°C prior to use. A single batch of PAI–749 was used for all studies.

2.2.2 TISSUE PLASMINOGEN ACTIVATOR

Aliquots of recombinant t-PA (Boehringer Ingelheim, Germany) for infusion into the extracorporeal circuit of the Badimon Chamber were prepared in 0.9% saline and stored at -80°C prior to use.

2.2.3 VASODILATORS

Acetylcholine, bradykinin (both supplied by MSD, UK) and sodium nitroprusside (David Bull Laboratories, UK) solutions for intra-arterial infusion were prepared in 0.9% saline on the day of use.

2.2.4 ENALAPRIL

Enalapril 10 mg (MSD, UK) and matched placebo were prepared and packaged by Tayside Pharmaceuticals, NHS Tayside, UK.

2.3 *EX VIVO* THROMBOSIS STUDIES

Ex vivo thrombus formation was measured using the Badimon Chamber. A 17-gauge venous cannula was inserted into a large subcutaneous vein in the antecubital fossa. A peristaltic pump (Masterflex model 7013, Cole-Palmer Instruments, USA), positioned distal to the chambers, was used to draw blood from the venous cannula via a length of polyethylene tubing, through a series of three cylindrical perfusion chambers maintained at 37˚C in a water bath (Figure 2.1). Each study lasted for five minutes during which blood flow was maintained at a constant rate of 10 mL/min. Carefully prepared strips of porcine aorta (Pel-Freez Biologicals, USA), from which the intima and a thin layer of media had been removed, acted as the thrombogenic substrate [Fernandez-Ortiz*, et al.*, 1994]. The rheological conditions in the first chamber simulate those of patent coronary arteries (low shear rate, approximately 212 s^{-1}), while those in the second and third chambers simulate those of mildly stenosed coronary arteries (high shear rate, approximately 1690 s^{-1}). Following perfusion of blood, the chambers were flushed with 0.9% saline for one minute under the same rheological conditions to remove residual blood and non-attached cells. All studies were performed using the same perfusion chambers. Immediately after each study, the porcine strips with thrombus attached were removed and fixed in 4% paraformaldehyde for 72 hours at 4˚C prior to being prepared for histological analysis.

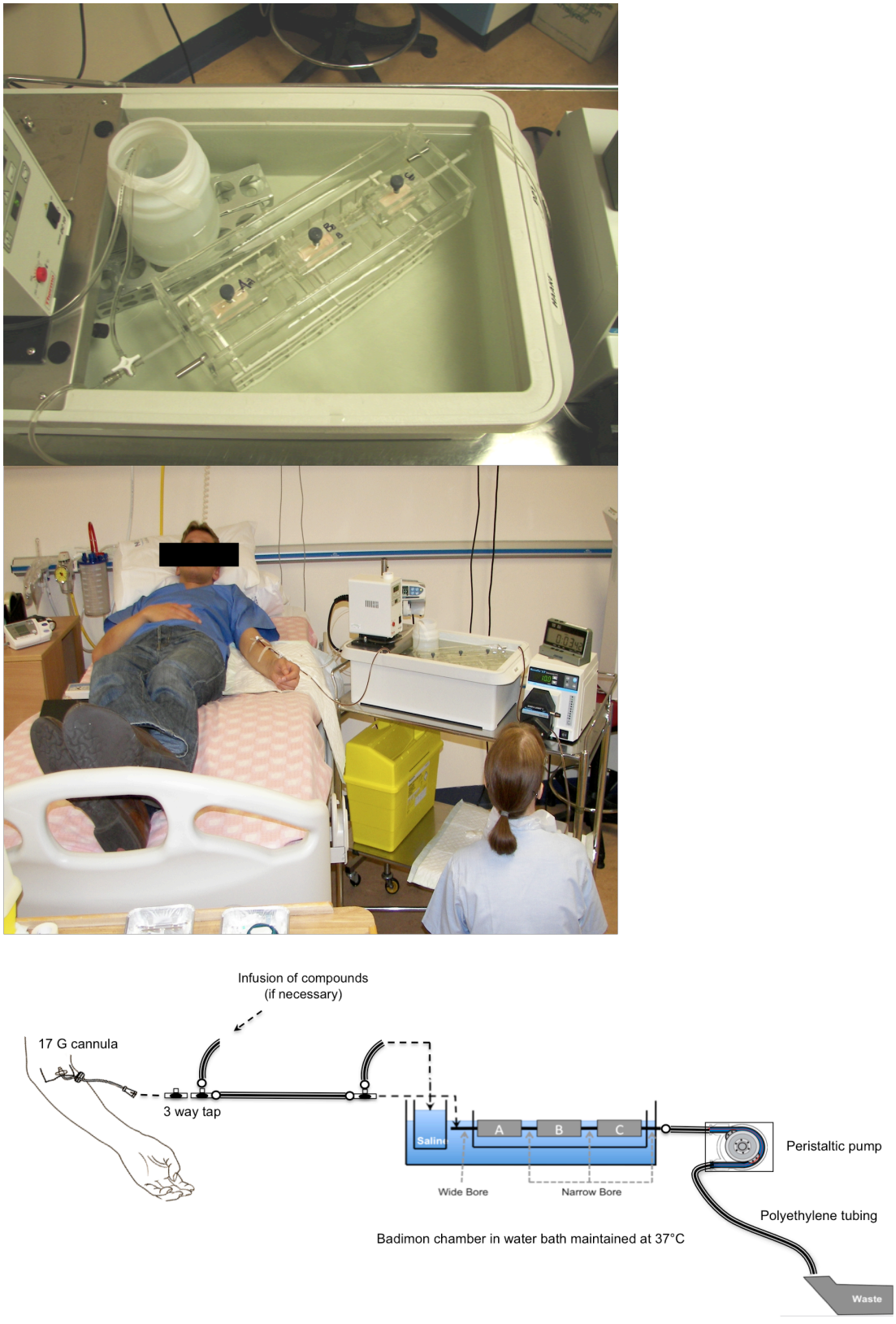

Figure 2.1 *The Badimon Chamber in use.*

2.3.1 ADDITION OF COMPOUNDS TO THE EXTRACORPOREAL CIRCUIT

For studies requiring the addition of compounds to the extracorporeal circuit, study drugs were added using a calibrated syringe driver (Alaris Arsena GH, Cardinal Health, USA) and allowed to mix prior to entering the perfusion chambers (Figure 2.1). Appropriate samples were taken from the effluent of the chamber in order to confirm accurate addition of compounds.

2.3.2 HISTOLOGICAL ANALYSIS

As thrombus forms along the entire length of the exposed porcine aortic strip, the cross-sectional area gives a reliable reflection of total thrombus [Dangas*, et al.*, 1998]. Following fixation, the proximal and distal 1 mm of the exposed substrate were removed and discarded. The remainder was cut into four pieces. These pieces were subsequently paraffin-wax embedded and 5 μm sections were prepared from each in order that quantification was performed on sections taken from all parts of the exposed tissue strip.

Sections were stained with Masson's trichrome stain to detect total thrombus or with an anti-fibrin II β chain mouse monoclonal antibody (clone T2G1; Accurate Chemical & Scientific Corporation, USA) to detect fibrin. For immunohistochemical staining, endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide and serum free protein block (Dako, Denmark). Sections were then incubated with the primary antibody at a final concentration of 10 μg/mL for 1 hour at room temperature. Detection was performed using EnVision™ (Dako, Denmark) and treatment with 3,3'-Diaminobenzidine substrate chromogen (Dako, Denmark). Finally, sections were

counterstained with haematoxylin. No staining was present on specimens not incubated with the primary antibody or on areas of the specimens not exposed to blood flow.

A purpose-designed semi-automated scanning microscope (BX61, 142 Olympus, UK) and image analysis system (Ariol 3.1, Applied Imaging, USA) were used to quantify thrombus area and composition. Digital images of each section were acquired at ×20 magnification. High-resolution classifiers, based on colour and shape parameters, were established to detect total thrombus and fibrin staining. Results from at least six sections were averaged to determine thrombus area for each chamber as described previously [Lev*, et al.*, 2006; Wahlander*, et al.*, 2006; Zafar*, et al.*, 2007].

2.4 FLOW CYTOMETRIC ASSESSMENTS OF PLATELET ACTIVATION

Samples were obtained directly from the venous cannula, from the tubing immediately before the perfusion chambers and from the effluent immediately after the perfusion chambers, as appropriate, and processed according to previously described protocols [Harding*, et al.*, 2006]. Blood was anticoagulated with D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (PPACK, 75 μm; Cambridge Biosciences, UK). Five minutes after sample collection, samples were stained with the following conjugated monoclonal antibodies, as appropriate: PE-conjugated CD14 (Dako, Denmark), PE-conjugated CD154, PE-conjugated CD62P (Becton-Dickinson, UK); PE-conjugated CD11b, PE-conjugated CD40, FITC-conjugated CD42a, FITC-conjugated CD14 (Serotec, USA); and appropriate control isotypes. All antibodies were diluted 1:20. Once stained, samples were incubated for 20 minutes at room temperature to identify P-selectin and CD40L on the platelet surface and CD40 on the monocyte surface. Platelet-monocyte samples were fixed with FACS-Lyse (Becton-Dickinson, UK); platelet samples were fixed with 1% paraformaldehyde. Samples were analysed within 24 hours using a FACScan flow cytometer (Becton-Dickinson, UK). Platelet–monocyte and platelet–neutrophil aggregates were defined as monocytes or neutrophils positive for CD42a, as described previously [Harding*, et al.*, 2007]. Analysis was performed using FlowJo (Treestar, USA).

2.5 DIESEL EXHAUST EXPOSURES

The diesel exhaust exposures were standardised to ensure a particle matter (PM) concentration *circa* 350 μg/m^3. These concentrations are found on a regular basis in heavy traffic, occupational settings, and in the world's most-polluted cities.

2.5.1 DIESEL EXHAUST EXPOSURES IN EDINBURGH, UK

Exposures in Edinburgh, UK were performed using a mobile ambient particle concentrator exposure laboratory (MAPCEL) in collaboration with a group from the Dutch National Institute of the Environment (RIVM).

During exposures, subjects performed moderate exercise (minute ventilation 25 L/min/m^2) on a bicycle ergometer for 15 minutes alternated with 15-minute rest periods. Temperature and humidity in the chamber were controlled at 22ºC and 50% respectively. Diesel exhaust was generated by an idling engine (type F3M2011, 2.2 L, 500 rpm; Deutz, Germany) using gas oil (Petroplus Refining, UK). Over 90% of the exhaust fumes were shunted away, with the remainder being diluted with air and fed into the exposure chamber at a steady-state concentration. Air in the chamber was continuously monitored with exposures standardised using continuous measurement of nitrogen oxide (NO_x) concentrations to deliver a particulate concentration of 350 μg/m^3. There was little variation in particle mass (348±68 μg/m^3), particle number (1.2±0.1 x 10^6/cm^3), NO_x (0.58±0.03ppm), NO_2 (0.23±0.02ppm), NO (0.36±0.02ppm), CO (3.54±0.76ppm) and total hydrocarbon (2.8±0.1 μg/m^3) concentrations between exposures.

2.5.2 DIESEL EXHAUST EXPOSURES IN UMEA, SWEDEN

Exposures in Umeå, Sweden were performed using a well-characterised exposure facility in Umeå, Sweden. This facility has been used to study the effects of dilute diesel exhaust on lung inflammation and function in a range of healthy volunteers and patients with pulmonary disease since its inception in 1994.

The diesel exhaust was generated from an idling Volvo diesel engine (Volvo TD45, 4.5L, 4 cylinders, 680rpm) from Swedish Low Sulphur Gasoil E10 (Preem, Sweden). Over 90% of the exhaust was shunted away, and the remainder diluted with filtered air heated to 20^{o}C (humidity ~50%) before being fed into a whole body exposure chamber at a steady-state concentration (Figure 2.2).

 (a)

Figure 2.2 *(a) Idling diesel engine and (b) whole body exposure chamber for human exposure studies to dilute diesel exhaust.*

During exposures, subjects performed moderate exercise as described above. Exposures were standardised using continuous measurement of NO_x to deliver a particulate concentration of 350 $\mu g/m^3$. There was little variation in particle mass (330±12 $\mu g/m^3$), particle number (1.26±0.01 x $10^6/cm^3$), NO_x (2.78±0.03ppm), NO_2 (0.62±0.01ppm), NO (2.15±0.03ppm), CO (3.08±0.12ppm) and total hydrocarbon (1.58±0.16 $\mu g/m^3$) concentrations between exposures.

Air inside the chamber was continuously monitored for pollutants with exposures standardised using real time measurements of NO_x. Nitrogen dioxide and nitrogen oxide were measured online using a chemiluminescence technique (ECO Physics CLD 700 AL Med, Switzerland). A UNOR 610 infrared spectrophotometer (Maihak AG, Germany) was used for online monitoring of carbon monoxide. Total

hydrocarbon in the exposure aerosol was measured using a flame ionisation detection method (Hydrocarbon Analyser, Model 3-300, JUM Engineering Co., USA). Continuous registration of the number of particles <1 μm diameter/cm^3 was obtained with a condensation particle counter (TSI, USA). The size distribution of particulate in the exposure chamber was determined using a scanning mobility particle sizer (TSI, USA).

The size distribution of PM in the exposure chamber demonstrated a lognormal distribution, typically ranged from 20 to 120 nm, with a count median diameter of 54 nm (geometric standard deviation = 1.7 nm; Figure 2.3). The polycyclic aromatic hydrocarbons (PAHs) consisted of semi-volatile gaseous compounds with only a minor fraction (3.5%) present as particulate associated material, 0.04% of total PM and 0.06% of the PM organic fraction. The dominating PAHs were phenanthrene, fluorene, 2-methylfluorene, dibenzothiophene and different methyl-substituted phenanthrenes accounting for approximately 90% of total PAH.

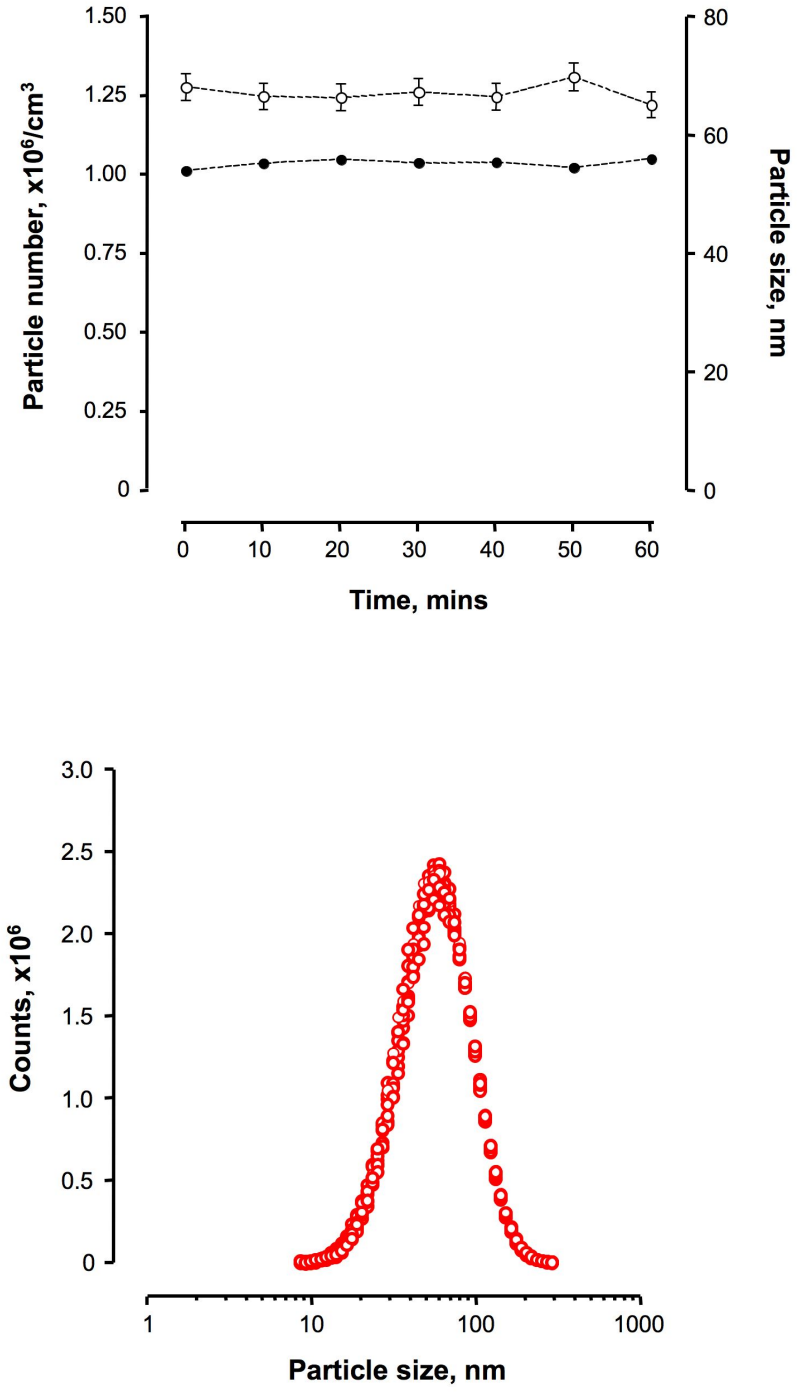

Figure 2.3 *a) Particle number (○) and count median diameter (●) of particulate during controlled exposure to dilute diesel exhaust for 60 minutes (mean±SEM, n=5). b) The size of inhaled diesel particulates (n=5) showed a lognormal distribution and typically ranged from 20 to 120 nm, with a count median diameter of 54 nm (geometric standard deviation = 1.7 nm).*

2.5.3 PARTICLE TRAP

The particle trap (DPF-CRT® [Continuously Regenerating Trap], Johnson Matthey, Royston, UK) used is an unmodified CRT filter, available commercially throughout the world as a factory-fit option or as a retrofit unit to buses and heavy goods vehicles (Figure 2.4). It is similar in design to filters produced by a number of manufacturers. It consists of a ceramic structure containing a honeycomb-like complex of channels through which the exhaust is passed. A platinum catalyst at the front of the filter oxidises part of the NO gas in the exhaust into NO_2, which flows through the particle filter and subsequently reacts with trapped carbonaceous particles to generate CO_2 and N_2. This increases NO_2 levels in the exhaust after the particle trap, without causing significant changes in total NO_x concentrations, whilst achieving an efficient reduction in particle emissions.

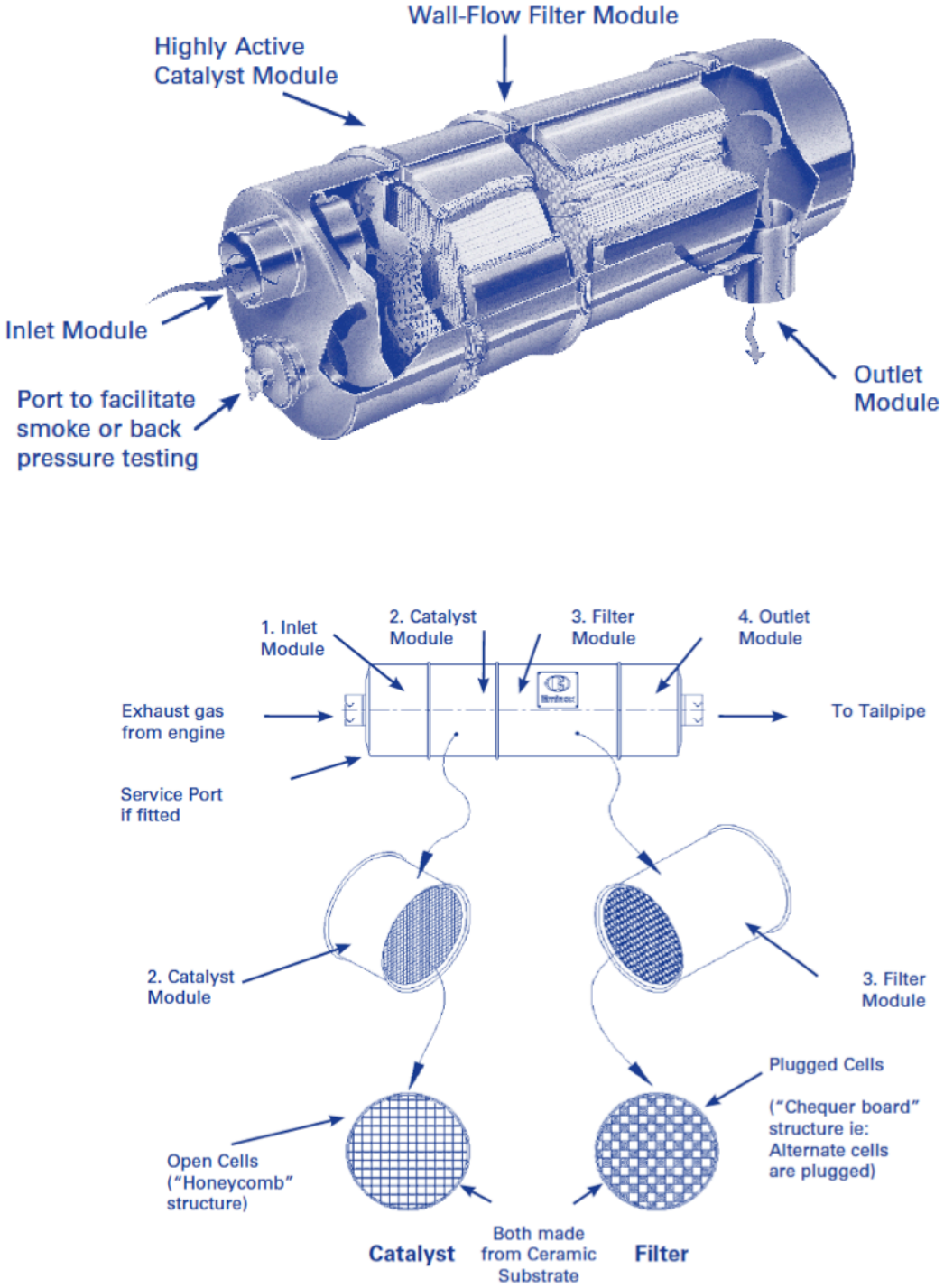

Figure 2.4 *The exhaust particle trap used in these studies was a continuously regenerating trap similar to that depicted above*

2.6 ASSESSMENT OF VASCULAR AND FIBRINOLYTIC FUNCTION

Measuring the response to vasoactive substances released by, or those that interact with, the vascular endothelium in the forearm is a useful measure of endothelial function. Local intra-arterial drug infusion into the brachial artery permits the direct assessment of vascular responses in the forearm without invoking concomitant effects on other organs. In this way the vessels are studied in their physiological environment under the influence of neuronal, circulating, and local mediators [Benjamin*, et al.*, 1995]. Although measurement of coronary vascular response is of greatest clinical relevance, invasive coronary studies can only really be performed in patients undergoing angiography. The close correlation between coronary and peripheral endothelium-dependent responses [Anderson*, et al.*, 1995] suggests that endothelial dysfunction may be a systemic state or that circulating factors have parallel effects in both coronary and peripheral arteries [Vita*, et al.*, 2004].

2.6.1 VENOUS OCCLUSION PLETHYSMOGRAPHY

This method of assessing resistance vessel function in the forearm is based on the principle of strain gauge venous impedance plethysmography. This technique examines the change in forearm blood flow (FBF) during intra-arterial (brachial artery) administration of agonists at locally active doses (Figure 2.5) [Benjamin*, et al.*, 1995; Webb, 1995].

Figure 2.5 *a) Intra-brachial artery infusions along with venous occlusion (upper arm) and supra-systolic pressure (wrist) cuffs in a healthy volunteer. b) A venous cannula for blood sampling is sited in the antecubital vein.*

The technique of venous occlusion plethysmography relies on intermittently preventing venous drainage from the arm using upper arm cuffs inflated to above venous pressure whilst arterial inflow is unaltered: blood can enter the forearm but cannot escape. This results in a linear increase in forearm volume over time, which is proportional to arterial blood inflow (Figure 2.6). Under resting conditions, approximately 70% of total forearm blood flow is through skeletal muscle. As the hand contains a high proportion of arteriovenous shunts with a different pharmacology and physiology it is excluded from the circulation by the application of inflation cuffs at supra-systolic pressure during the measurement. The technique of bilateral forearm blood flow measurement is highly reproducible within individuals [Walker*, et al.*, 2001] and is ideally suited to assessment of interventional strategies with repeated measurements [Wilkinson*, et al.*, 2001].

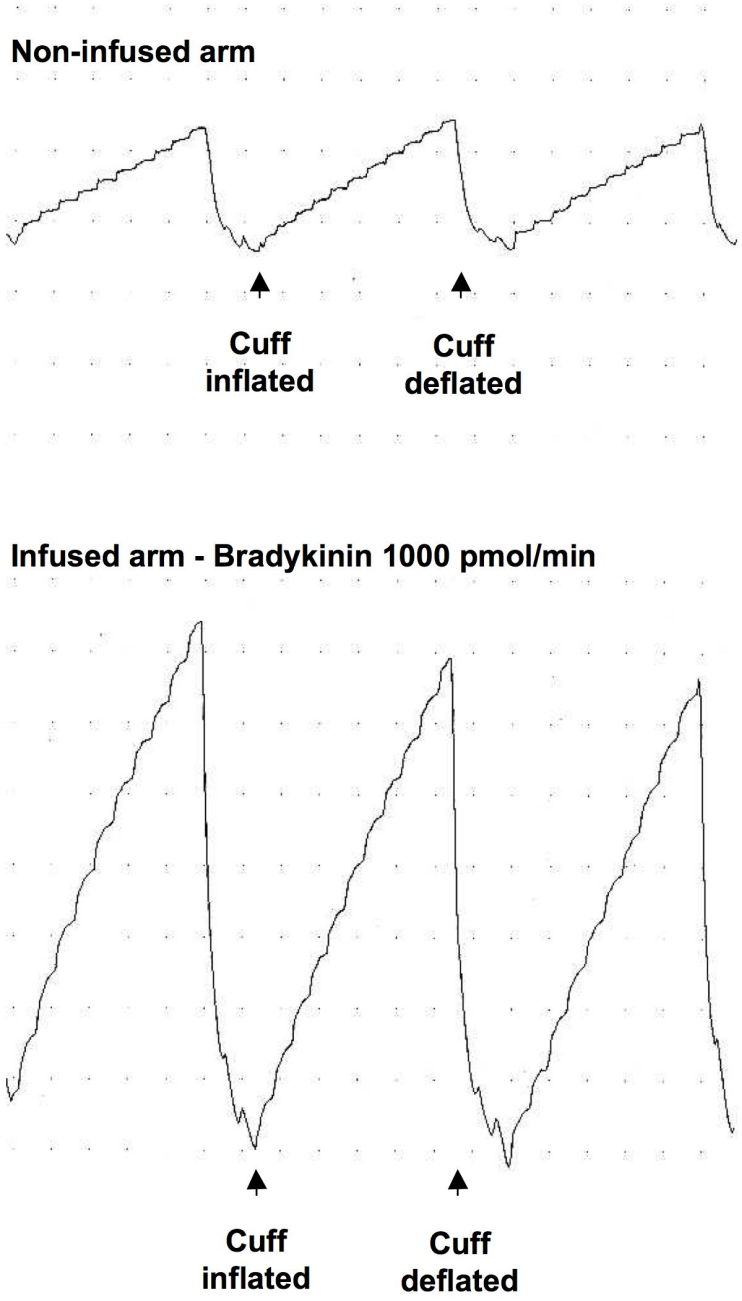

Figure 2.6 *Typical blood flow recording from non-infused and infused arms with infusion of intra-brachial bradykinin during venous plethysmography study.*

Brachial Artery Cannulation

The brachial artery of the non-dominant arm was cannulated with a 27-gauge steel needle (Cooper's Needle Works Ltd, UK) under 1% lidocaine (Xylocaine; Astra Pharmaceuticals Ltd, UK) local anaesthesia. The cannula was attached to a 16-gauge epidural catheter (Portex Ltd, UK) and patency maintained by infusion of saline (0.9%; Baxter Healthcare Ltd, UK) via an IVAC P6000 syringe pump (IVAC Ltd., UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 mL/min.

Blood Flow Measurement

Blood flow was measured in the infused and non-infused forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges that were applied to the widest part of the forearm [Benjamin*, et al.*, 1995]. Both arms were placed above the level of the right atrium and upper arm cuffs were inflated intermittently to 40 mmHg (venous occlusion) pressure for 10 seconds in every 15 seconds to achieve venous occlusion and obtain plethysmographic recordings. During measurement periods, the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mmHg (supra-systolic) using E20 Rapid Cuff Inflators (D.E. Hokanson Inc., Washington, USA). Analogue voltage output from an EC-4 strain gauge plethysmograph (D.E. Hokanson Inc., USA) was processed by a PowerLab® analogue-to-digital converter and Chart™ v5.0.1 software (AD Instruments Ltd., UK) and recorded onto a Dell Latitude® laptop (Dell Computers Ltd., UK). Calibration was achieved using the internal standard of the plethysmograph. Heart rate and blood pressure were monitored in the non-infused arm throughout each study with a non-invasive, semi-automated oscillometric sphygmomanometer (Boso Medicus, Germany).

Plethysmographic Data Analysis

A single operator undertook analysis of all plethysmographic data collected during the forearm studies in a blinded manner. Forearm blood flow responses are reported as absolute blood flow responses (mL/100 mL tissue/min) in the infused and non-infused arm. Plethysmographic data were extracted from the Chart™ data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel v12.3, Microsoft Corp., USA). Recordings from the first 60 seconds after wrist cuff inflation were not used because of the variability in blood flow during this period [Benjamin*, et al.*, 1995]. Usually, the last five flow recordings in each 3-minute measurement period were calculated and averaged for each arm.

2.7 VENOUS SAMPLING AND LABORATORY ASSAYS

2.7.1 SAMPLE PREPARATION AND PROCESSING

Differential white cell count, haematocrit, clinical biochemical assays, prothrombin and activated partial thromboplastin times were undertaken on fresh venous samples in Edinburgh (Departments of Haematology and Clinical Biochemistry, Lothian NHS University Hospitals Trust, UK) and Umeå (Departments of Haematology and Clinical Biochemistry, Umeå Hospital University, Sweden).

Citrate and acidified buffered citrate samples (Stabilyte^{TM}, Biopool International, USA; for fibrinolytic components) were kept on ice before being centrifuged at 2,000*g* for 30 minutes at 4°C. Ethylenediaminetetraacetic acid samples (BD Vacutainer, USA; for PAI–749 assays) were kept at room temperature before being centrifuged at 1,000*g* for 10 minutes at 20°C. Serum samples were centrifuged at 2,000*g* for 20 minutes after being allowed to clot on ice. Following centrifugation, platelet free plasma or serum was decanted and stored at -80ºC before assay.

2.7.2 FIBRINOLYTIC AND INFLAMMATORY ASSAYS

Plasma t-PA antigen and activity (t-PA Combi Actibind Elisa Kit, Technoclone, Austria), PAI-1 antigen and activity (Zymutest PAI-1 antigen and Zymutest PAI-1 Activity, Hyphen Biomed, France), D-dimer (Asserachrom®, Roche, Switzerland) and thrombin-antithrombin (TAT) complex (Enzgnost, Dade Behring Inc., UK) were determined by commercially available enzyme linked immunosorbant assays (ELISA). Intra-assay coefficients of variation were 7.0% and 5.5% for t-PA and PAI-1 antigen, 4.0% and 2.4% for t-PA and PAI-1 activity, 4.2% for D-dimer and 6.1% for

TAT complex, respectively. Inter-assay coefficients of variability were 4.0%, 7.3%, 4.0%, 7.6%, 6.0% and 4.5%, respectively. The sensitivities of the assays were 0.5 ng/mL, 0.10 IU/mL, 2.5 ng/mL, 5 IU /mL, 2.0 ng/mL and 2.2 ng/mL respectively. Measurement of t-PA/PAI-1 complex was undertaken as described previously [Bennett*, et al.*, 1990].

Plasma IL-6, tumour necrosis factor-alpha (TNF-α), soluble P-selectin, and soluble intracellular adhesion molecule-1 (ICAM-1) were measured with commercially available ELISAs (Quantikine, R&D Systems). Intra-assay, and inter-assay coefficients of variability were 5.0% and 6.9%, and 4.3% and 6.6% for plasma TNF-α and IL-6 respectively. Serum CRP concentrations were determined using with a validated highly sensitive assay (Department of Clinical Biochemistry; Fife NHS Trust, UK) using the method of particle-enhanced immunonephelometry (Behring BN II nephelometer, Dade Behring Inc., UK). Intra-assay and inter-assay coefficients of variability for hs-CRP were 3.3% and 4.9% respectively.

All assays were performed in duplicate and the mean value taken.

2.8 DATA ANALYSIS AND STATISTICS

Continuous variables are reported as mean ± standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism (GraphPad Software, USA) using paired Student's *t*-tests, analysis of variance (ANOVA) with repeated measures and calculation of the Pearson correlation coefficient as appropriate. Statistical significance was taken at the 5% level. Reproducibility was assessed by the method of Bland & Altman [Bland*, et al.*, 1986] and coefficients of reproducibility calculated for 95% confidence intervals.

CHAPTER 3

CHARACTERISATION AND REPRODUCIBILITY OF A HUMAN EX VIVO MODEL OF THROMBOSIS

Published by **Lucking AJ**, Chelliah R, Trotman AD, Connolly TM, Feuerstein GZ, Fox KA, Boon NA, Badimon JJ, Newby DE. Characterisation and reproducibility of a human *ex vivo* model of thrombosis
Thromb Res. 2010 Nov; 126(5):431-5.

3.1 SUMMARY

The Badimon chamber is a clinical *ex vivo* model of thrombosis that mimics flow conditions within the coronary circulation of man. The aims of this study were to characterise thrombus formation in the chamber and evaluate its reproducibility. In 24 healthy volunteers, total thrombus area was assessed at low and high shear rates with porcine aortic tunica media as the thrombogenic substrate. Reproducibility of thrombus formation was assessed by paired measurements made both within and between days. Platelet activation was assessed by flow cytometry, and fibrin content and distribution were assessed by immunohistochemistry. Thrombus area was highly reproducible in the low ([mean thrombus area, mean difference ± SEM] 8,018 μm^2, 58±204 μm^2 and 8,177 μm^2, -154±168 μm^2 within and between days respectively) and high shear chambers (11,802 μm^2, -52±175 μm^2 and 11,877 μm^2, 220±181 μm^2 within and between days respectively). Total thrombus area was greater in the high compared to the low shear chamber (11,970±285 μm^2 *versus* 7,892±298 μm^2; $P<0.0001$). Thrombus within the low shear chamber contained a greater proportion of fibrin (25.0±6.0% *versus* 8.3±1.6% in high shear chamber, $P<0.001$). Platelet activation was only detected once blood had passed through the chamber ($P=0.049$ for platelet-monocyte aggregate formation and $P=0.078$ for P-selectin expression). There was no association between basal platelet activation and thrombus area ($P=0.85$ and $P=0.66$ in the low and high shear chamber respectively).

The Badimon chamber provides a highly reproducible technique for the assessment of *ex vivo* thrombus formation in man.

3.2 INTRODUCTION

Cardiovascular disease is the leading cause of death worldwide with thrombosis playing a central role in its pathogenesis, particularly during acute events such as myocardial infarction. Whilst preclinical models can provide useful insights into novel antithrombotic interventions, species-specific differences ensure that phase I clinical studies remain an essential part of antithrombotic drug development [Leadley*, et al.*, 2000].

The demonstration of antithrombotic efficacy in man is challenging, Most techniques involve the evaluation of specific plasma and cellular components in isolation, require the addition of an anticoagulant to limit *in vitro* activation, and are often performed under static conditions. In contrast, *in vivo* thrombus initiation and growth occur in whole blood, under conditions of continuous flow, and often in the presence of vascular injury. Thus assessment of the efficacy of antiplatelet and anticoagulant agents may not be reflected by static or unidimensional systems employed by *in vitro* assays [Kroll*, et al.*, 1996]. An *in vivo* model for use in clinical studies presents significant safety issues and does not currently exist. The Badimon chamber is an *ex vivo* model of thrombosis that is suitable for use in clinical studies and has previously been used to assess novel antithrombotic regimens [Lev*, et al.*, 2006; Wahlander*, et al.*, 2006; Zafar*, et al.*, 2007; Zafar*, et al.*, 2007]. It has a number of important advantages over other techniques including the ability to assess thrombus formation on a pathophysiologically relevant substrate and under conditions of continuous flow [Badimon*, et al.*, 1986; Badimon*, et al.*, 1987].

Although the Badimon chamber technique is well-established, previous characterisation studies were performed in a porcine system and using methodology that has since been superseded [Badimon*, et al.*, 1986; Badimon*, et al.*, 1987]. In addition, there are no published studies specifically addressing the reproducibility of thrombus formation in the chamber. Therefore, the principal aims of this study were to characterise the thrombus formed in the chamber, and to assess within and between day reproducibility. Specifically, we used a semi-automated combined image acquisition and analysis system to measure total thrombus area and the contribution of fibrin. In addition, the potential for platelet activation to occur within the extracorporeal circuit may limit the sensitivity of the technique to detect subtle changes in thrombotic potential, particularly in situations where platelet activation is likely to be a major determinant of thrombus formation. Using flow cytometry, we therefore assessed the effect of blood transit through the extracorporeal circuit and the perfusion chambers on platelet activation. Finally we investigated whether basal levels of platelet activation were related to thrombus area.

3.3 METHODS

3.3.1 SUBJECTS

Twenty-four healthy non-smokers aged between 18 and 30 years old were enrolled into the study that was performed with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and the written informed consent of all volunteers. Exclusion criteria were bleeding diasthesis, the use of regular medication or any clinically significant illness.

3.3.2 *EX VIVO* THROMBOSIS STUDIES

Thrombus formation was measured using the Badimon Chamber as described in section 2.3.

Histological Analysis

Sections were stained with Masson's trichrome stain to detect total thrombus or with an anti-fibrin II β chain mouse monoclonal antibody (clone T2G1; Accurate Chemical & Scientific Corporation, USA) to detect fibrin. For immunohistochemical staining, endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide and serum free protein block (Dako, Glostrup, Denmark). Sections were then incubated with the primary antibody at a final concentration of 10 μg/mL for 1 hour at room temperature. Detection was performed using EnVision™ (Dako, Denmark) and treatment with 3,3'-Diaminobenzidine substrate chromogen (Dako, Denmark). Finally, sections were counterstained with haematoxylin. No staining was present on specimens not incubated with the primary antibody or on areas of the specimens not exposed to blood flow.

A purpose-designed semi-automated scanning microscope (BX61, 142 Olympus, UK) and image analysis system (Ariol 3.1, Applied Imaging, USA) was used to quantify thrombus area and composition. Digital images of each section were acquired at ×20 magnification. High-resolution classifiers, based on colour and shape parameters, were established to detect total thrombus, platelet and fibrin staining. Results from at least six sections were averaged to determine thrombus area for each chamber as described previously [Lev*, et al.*, 2006; Wahlander*, et al.*, 2006; Zafar*, et al.*, 2007].

3.3.3 FLOW CYTOMETRY

Samples were obtained directly from the venous cannula, from the tubing immediately before the perfusion chambers and from the effluent immediately after the perfusion chambers and processed according to previously described protocols [Harding*, et al.*, 2006]. In brief, blood was anticoagulated with D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (PPACK, 75 μm; Cambridge Biosciences, UK). Five minutes after sample collection, samples were stained with the following conjugated monoclonal antibodies: PE-conjugated CD14 (Dako, Denmark), PE-conjugated CD62P (Becton-Dickinson, UK); PE-conjugated CD11b, FITC-conjugated CD42a, FITC-conjugated CD14 (Serotec, USA); and appropriate control isotypes. All antibodies were diluted 1:20. Once stained, samples were incubated for 20 minutes at room temperature. Platelet-monocyte samples were fixed with FACS-Lyse (Becton-Dickinson, UK); platelet samples were fixed with 1% paraformaldehyde. Samples were analysed within 24 hours using a FACScan flow cytometer (Becton-Dickinson, UK). Platelet–monocyte aggregates were defined as

monocytes staining positive for CD42a, as described previously [Harding*, et al.*, 2007]. Analysis was performed using FlowJo (Treestar, USA).

3.3.4 STUDY DESIGN

To assess within and between day reproducibility, sixteen subjects attended on two separate occasions, two weeks apart. On the first visit, two studies were performed 45 minutes apart followed by a final study on the second visit. Samples for flow cytometry were taken during the first visit in eight of the subjects. A separate cohort of eight subjects attended for a single study to allow the specific assessment of fibrin area and distribution within the thrombi. All 24 subjects attended fasted at 9 am on the day of the study. All abstained from alcohol for 24 hours and from food and caffeine-containing drinks for at least six hours before each study. Studies were carried out with subjects lying supine in a quiet, temperature-controlled room maintained at 22°C to 25°C.

3.3.5 STATISTICAL ANALYSIS

Continuous variables are reported as mean ± standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism (GraphPad Software, USA) using paired Student's *t*-tests, analysis of variance (ANOVA) with repeated measures and calculation of the Pearson correlation coefficient as appropriate. Statistical significance was taken at $P<0.05$. Reproducibility was assessed by the method of Bland & Altman [Bland*, et al.*, 1986] and coefficients of reproducibility calculated for 95% confidence intervals.

3.4 RESULTS

All study visits were completed in each subject and were well tolerated.

3.4.1 REPRODUCIBILITY

Thrombus area was highly reproducible in the low and high shear chambers, both within and between days (Figures 3.1 and 3.2; Table 3.1). The 95% confidence intervals, indicated by the coefficients of reproducibility, are similar within and between days indicating that thrombus formation assessed in the Badimon Chamber is consistent over time within this healthy population.

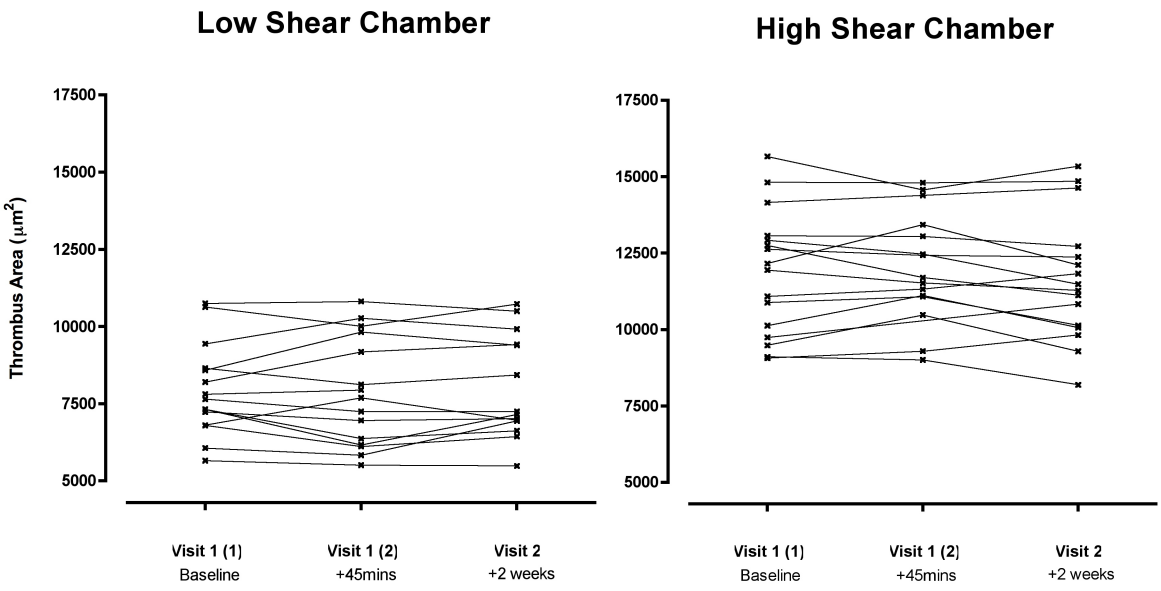

Figure 3.1 *Within and between day reproducibility of ex vivo thrombus formation (n=16).*

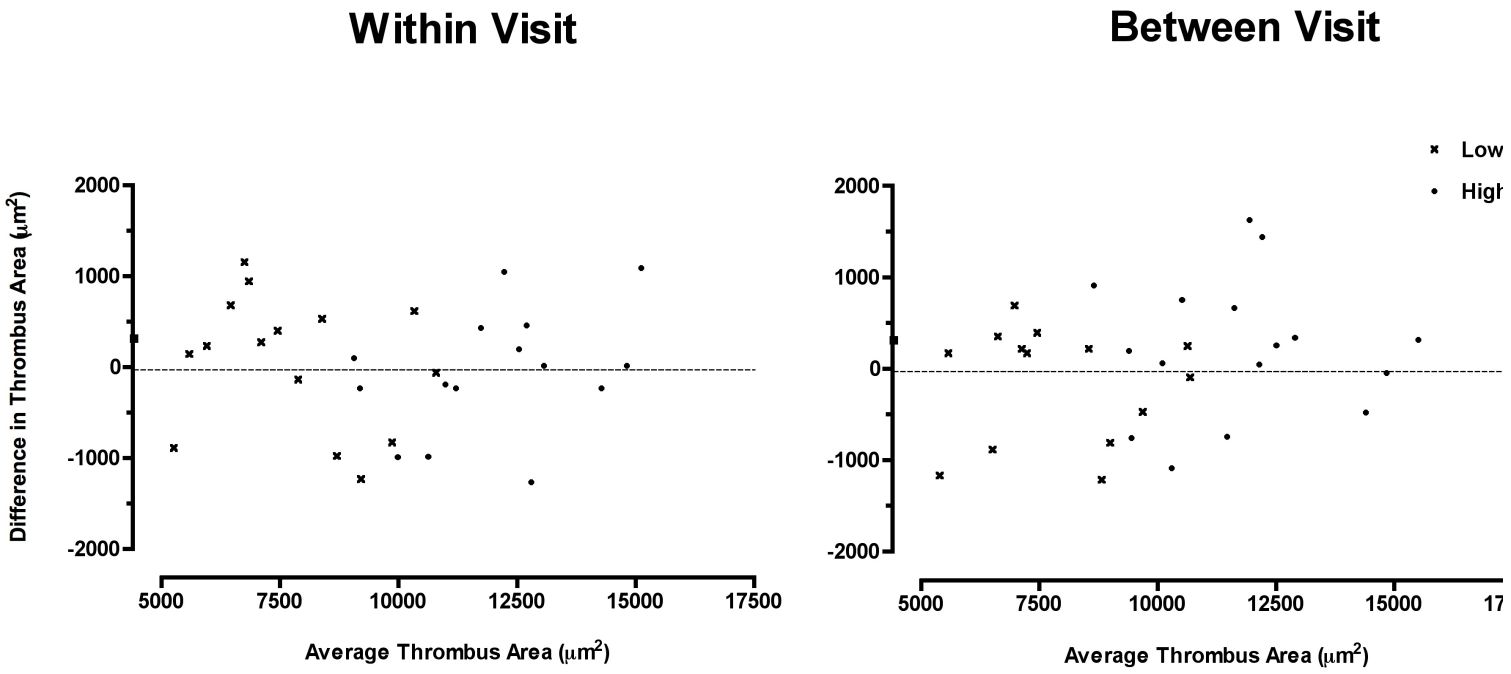

Figure 3.2 *Bland-Altman plots of thrombus formation within and between days (n=16).*

	Within day reproducibility			
	Mean thrombus area (μm^2)	*Mean of the differences*	*Coefficient of reproducibility*	*Mean thromb area (μm^2)*
Low Shear Chamber	8018	58	1443	8177
High Shear Chamber	11802	-52	1331	11877

Table 3.1 *Within and between day reproducibility of* ex vivo *thrombus formation*

3.4.2 QUANTIFICATION AND DISTRIBUTION OF FIBRIN

Total thrombus area was greater in the high compared to the low shear chamber (12,730±550 μm^2 *versus* 7,401±512 μm^2, P<0.0001; Figure 3.3a). Although absolute fibrin area appeared only marginally greater in the low shear chamber (2,347±129 μm^2 *versus* 1,877±233 μm^2, P=0.068; Figure 3.3a), fibrin formed a substantially greater proportion of the thrombus within the low shear chamber compared to the high shear chamber (25.0±6.0% *versus* 8.3±1.6%, P<0.001). The fibrin was predominantly distributed along the base of the thrombus, with platelets aggregates forming above this layer of fibrin (Figure 3.3b).

3.4.3 PLATELET ACTIVATION

Consistent with a healthy volunteer cohort, baseline levels of platelet-monocyte aggregates (22±2.8%) and platelet P-selectin expression (0.42±0.12%) were low [Harding*, et al.*, 2007]. Whilst levels of platelet-monocyte aggregates and platelet P-selectin expression appeared to increase as blood passed across the perfusion chambers (P=0.049 and P=0.078 respectively; Figure 3.4), tubing in the extracorporeal circuit did not cause platelet activation. There was no correlation between baseline levels of platelet-monocyte aggregates and thrombus formation in either the low or high shear chambers (r=-0.12, P=0.85 and r=-0.23, P=0.66 respectively).

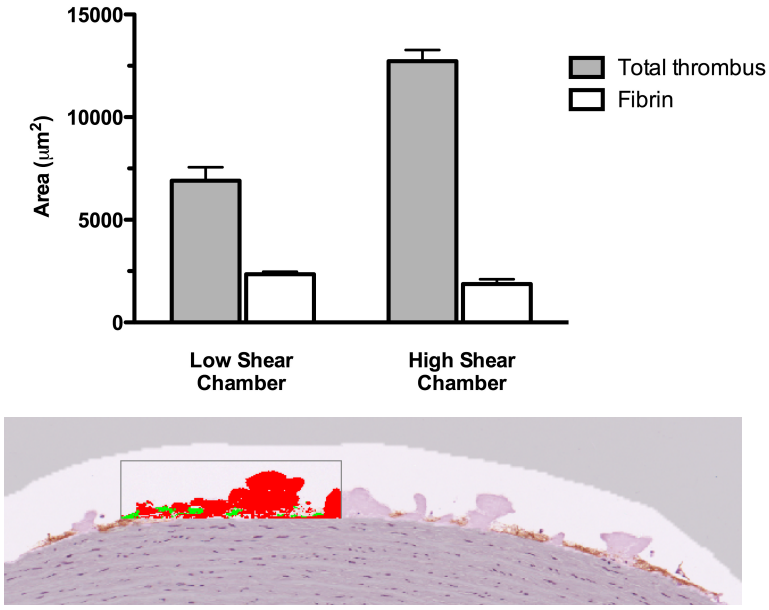

Figure 3.3 a) *Total thrombus area and fibrin area within the low and high shear chambers. Data shown are mean ± SEM (n=8).* ***b)*** *Representative photomicrograph of porcine media exposed to flowing blood in the high shear chamber, demonstrating the method used to quantify thrombus formation. This section has been stained for fibrin and counterstained with haematoxylin. Staining reveals two morphologically distinct components within the thrombus; irregularly shaped platelet aggregates (light pink) and a more uniform, fibrin layer that forms along the surface of the porcine media (brown). The rectangular box indicates an area marked out for analysis. The software is calibrated using colour to differentiate between areas of fibrin (highlighted in green) and areas of platelet-rich thrombus (highlighted in red). Total thrombus area was calculated by adding fibrin area to platelet-rich area. Original magnification × 20.*

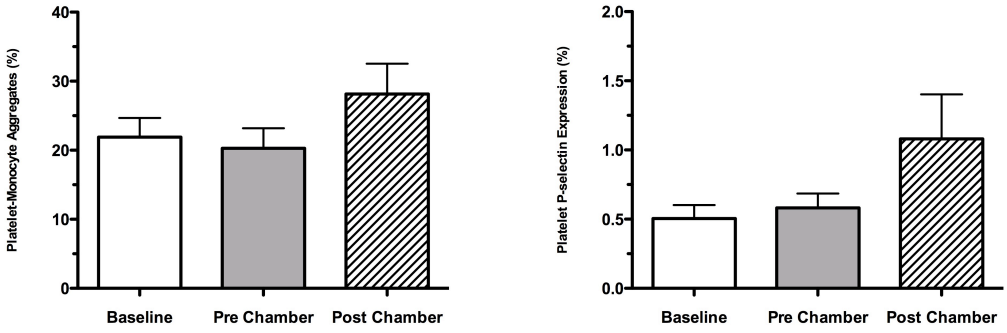

Figure 3.4 *The effect of passage through the extracorporeal circuit and across the perfusion chambers on platelet activation. Data shown are mean ± SEM (n=8).*

3.5 DISCUSSION

The Badimon chamber produces a platelet-rich thrombus that forms upon a basal layer of fibrin. The contribution of fibrin is greater under conditions of lower shear stress, whilst platelets contribute more under conditions of high shear stress. We have here found that measurement of the total area of this type of thrombus is highly reproducible both within and between days. We suggest that this technique provides a flexible clinical *ex vivo* model of thrombosis suitable for use in crossover interventional studies.

3.5.1 REPRODUCIBILITY

The reproducibility of the Badimon chamber in human subjects has not been previously assessed and is an important aspect of validating the technique. In addition, several important changes to the methodology have recently been made, necessitating re-evaluation of the technique. Here, we found that thrombus formation within the low and high shear chambers was highly reproducible both within and between days with similar coefficients of reproducibility. This suggests that the Badimon Chamber provides a robust and reproducible means of assessing whole blood thrombogenicity that is valid for use in multi-visit crossover studies.

Whilst blood flow within this system is not pulsatile, rheological conditions otherwise broadly simulate those at the site of intra-coronary atheromatous plaque rupture. In combination with the use of porcine tunica media as a pathophysiologically relevant thrombogenic substrate, the technique serves as a model of deep coronary arterial injury. As such, we believe that it is particularly useful for evaluating the efficacy of novel antithrombotic interventions.

Previous preclinical studies evaluating the Badimon chamber included several important differences to its current application in clinical studies. First, many studies were performed in pigs using heparinised blood from the carotid artery passing through the extracorporeal circuit containing the perfusion chambers, before returning to the animal via the jugular vein. These studies also evaluated porcine aortas in which selective endothelial injury had been caused without disruption to the underlying basement membrane. Moreover, initial studies used platelet deposition (measured using ^{111}Indium-labelled platelets) as a surrogate for thrombus formation. Thus, many distinct differences exist that necessitate critical evaluation in its application to the clinical setting.

Given the safety and logistical difficulties of using radiolabelled blood components, a histological method for assessing thrombus area was developed for clinical studies. Whilst more time-consuming, histological analysis has several important advantages over ^{111}Indium-labelled platelets. By using appropriate conventional staining techniques in combination with immunohistochemistry, total thrombus area and specific components can be quantified [Dangas*, et al.*, 1998; Giesen*, et al.*, 1999]. For the majority of studies, Masson's trichrome stain is sufficient to quantify both fibrin-rich and platelet-rich thrombus. Histological assessment also allows for the identification of confounding factors such as those caused by gross surface irregularities and flaps that can occur on the denuded aortic strips. Here thrombus formation will be driven by tissue disruption rather than the thrombotic state of the subject or the proposed drug intervention.

In this study we utilised a validated purpose-designed imaging system capable of combined semi-automated image acquisition and analysis coupled with a slide loading system [Gokhale*, et al.*, 2007]. This contrasts with other work where image data were acquired using manual camera/microscope stages before being transferred and analysed using separate image analysis software. Given the large amount of image data produced during these studies, the use of an integrated system improves the efficiency and accuracy of the analysis process, and minimises the potential for errors to be made during image acquisition.

Although the majority of previous studies have used a parallel group design, we here specifically addressed the suitability of the technique for use in multi-visit crossover studies by assessing both between day as well as within day reproducibility. However, given that only healthy volunteers were used, we acknowledge that between day reproducibility, in particular, may not be as robust in a patient cohort where period and time-order effects caused by fluctuations in disease-activity and treatment-response may play a role; factors that were mitigated in this study.

3.5.2 CHARACTERISATION

Consistent with studies of arterial thrombus formation performed using real-time *in vivo* imaging in the mouse [Falati*, et al.*, 2002], fibrin was distributed in a uniform manner along the surface of the porcine aortic strip. The remainder of the thrombus is predominantly composed of platelets that project into the chamber lumen as irregular shaped aggregates. This distribution of fibrin and platelets is consistent with previous clinical reports of the Badimon chamber [Dangas*, et al.*, 1998]. This would suggest that a carpet of fibrin is formed on the surface of a disrupted vessel that then acts as a

platform for platelet aggregates to form. The proportionate contribution of fibrin to total thrombus area was greatest for the low shear chamber consistent with the greater role of platelets under conditions of high shear stress. This may have implications for the investigation of interventions likely to have a predominant effect on fibrin content.

3.5.3 PLATELET ACTIVATION

The extracorporeal circuit of the system comprises polypropylene tubing specifically selected in order to minimise activation of platelets and components of the coagulation system. It was reassuring that platelet activation did not occur within the extracorporeal circuit prior to contact with the perfusion chambers themselves. However, platelet activation did occur following passage through the perfusion chambers themselves, consistent with exposure to elevated shear conditions and the thrombogenic surface therein. The surface of the arterial media is comprised of a variety of subendothelial components including collagen types III and IV, fibronectin and smooth muscle cells [Dangas*, et al.*, 1998], all of which are know to activate platelets, principally through interaction with the platelet glycoprotein Ib receptor. Indeed, given exposure to these conditions, it might be expected that platelet activation would be greater. However, a large proportion of activated platelets may have become incorporated into the thrombus itself and were thus not detectable in the chamber effluent or alternatively the relatively short transit time across the chambers permitted only limited platelet activation or formation of platelet-monocyte aggregates.

Baseline levels of platelet activation did not correlate with thrombus area in either the low or high shear chambers. This suggests that in healthy volunteers, the principal

determinant of thrombus formation is the nature of the thrombogenic substrate rather than the state of platelet activation. However, we acknowledge that given the limited sample size combined with low baseline levels of platelet activation in a healthy volunteer cohort, this study was not adequately powered to detect any potential correlation; the chance of a type II error is thus high. Further studies are required in subjects and patients with a wider range of platelet activation.

3.5.4 CONCLUSION

We have demonstrated that *ex vivo* thrombus formation in the Badimon Chamber is highly reproducible when assessed within and between days in healthy volunteers. We believe that this technique provides a flexible model with which to assess novel and existing antithrombotic interventions using parallel group and crossover study designs.

CHAPTER 4

ENDOGENOUS TISSUE PLASMINOGEN ACTIVATOR ENHANCES FIBRINOLYSIS AND LIMITS THROMBUS FORMATION IN A CLINICAL MODEL OF THROMBOSIS

Lucking AJ, Gibson KR, Paterson EE, Faratian D, Ludlam CA, Boon NA, Fox KAA, Newby DE. Endogenous Tissue Plasminogen Activator Enhances Fibrinolysis and Limits Thrombus Formation in a Clinical Model of Thrombosis
Accepted 13th February 2012 for publication in *ATVB*

4.1 SUMMARY

Fibrinolysis inhibits thrombus propagation on the surface of unstable atherosclerotic plaques. Using a clinical model of deep arterial injury, we assessed the ability of exogenous and endogenous tissue plasminogen activator (t-PA) to limit acute *in situ* thrombus formation. *Ex vivo* thrombus formation was assessed in the Badimon Chamber at low and high shear rates in two double-blind randomised cross-over studies of 20 healthy volunteers during extracorporeal administration of recombinant t-PA (0, 40, 200 and 1000 ng/mL) or during endogenous t-PA release stimulated by intra-arterial bradykinin infusion in the presence or absence of oral enalapril. Recombinant t-PA caused a dose-dependent reduction in thrombus area under low and high shear conditions ($p<0.001$ for all). Intra-arterial bradykinin increased plasma t-PA concentrations in the chamber effluent ($p<0.01$ for all *versus* saline) that was quadrupled in the presence of enalapril ($p<0.0001$ *versus* placebo). These increases were accompanied by an increase in plasma D-dimer concentration ($p<0.005$ for all *versus* saline) and, in the presence of enalapril, a reduction in thrombus area in the low shear (16%±5%, $p=0.03$) and a trend towards a reduction in the high shear chamber (13%±7%, $p=0.07$).

Using a well-characterised clinical model of coronary arterial injury, we demonstrate that endogenous t-PA released from the vascular endothelium enhances fibrinolysis and limits *in situ* thrombus propagation. These data support a crucial role for the endogenous fibrinolytic system *in vivo* and suggest that continued exploration and manipulation of its therapeutic potential is warranted.

4.2 INTRODUCTION

The endogenous fibrinolytic system protects against intravascular fibrin formation and thrombosis, and appears to be particularly important within the coronary circulation [Rosenberg*, et al.*, 1999]. In the presence of a developing thrombus, tissue plasminogen activator (t-PA) is rapidly released from a dynamic storage pool within the vascular endothelium through its stimulation by various factors that include bradykinin, thrombin and factor Xa [van den Eijnden-Schrauwen*, et al.*, 1995]. The released t-PA causes a 1000-fold increase in the enzymatic conversion of plasminogen to plasmin [Fox*, et al.*, 1985; Tranquille*, et al.*, 1989]. This ensures that rapid plasmin generation, fibrin degradation and clot dissolution are tightly regulated and localised to sites of vascular injury. Thus, the rapidity and extent of acute t-PA release from the endothelium is a critical factor in determining the efficacy of local endogenous fibrinolysis and the prevention of intravascular thrombus propagation [Jern*, et al.*, 1994; Newby*, et al.*, 1997]. Whilst many workers have assessed basal plasma concentrations of t-PA and its principal inhibitor, plasminogen activator inhibitor type 1 (PAI-1), these do not reflect the capacity of the endothelium to release t-PA acutely nor the dynamic ability to dissolve intravascular thrombus.

Robust methods to determine acute t-PA release within the human forearm and coronary circulations have been developed [Newby*, et al.*, 2001; Newby*, et al.*, 1997]. In the forearm circulation, local t-PA release has been demonstrated in response to a variety of physiological and pharmacological stimuli including thrombin receptor agonism [Gudmundsdottir*, et al.*, 2006], substance P [Newby*, et al.*, 2002; Newby*, et al.*, 1997], tumour necrosis factor α [Chia*, et al.*, 2003] and bradykinin [Labinjoh*, et al.*, 2000]. Indeed, the fibrinolytic capacity of the endothelium predicts future adverse

cardiovascular events in patients with coronary disease and may provide additional insights into endothelial function [Robinson*, et al.*, 2007]. However, the primary end-point in all studies to date has been the quantification of t-PA release and it is unknown whether this acute endogenous release of t-PA can truly influence or modify dynamic thrombus formation.

The demonstration of antithrombotic efficacy in man is challenging. Most techniques evaluate specific plasma or cellular components in isolation and are performed under static conditions *in vitro*. In contrast, *in vivo* thrombus initiation and growth occur in whole blood, under conditions of continuous flow and in the presence of vascular injury. Thus the assessment of the efficacy of antithrombotic interventions may not be reflected by static or unidimensional systems employed by *in vitro* assays [Kroll*, et al.*, 1996]. An *in vivo* model for use in clinical studies presents significant safety issues and does not currently exist. The Badimon chamber is an *ex vivo* model of thrombosis with the ability to assess thrombus formation on a pathophysiologically relevant substrate under conditions of continuous flow [Badimon*, et al.*, 1986; Badimon*, et al.*, 1987]. It has previously been used to assess novel antithrombotic interventions [Lev*, et al.*, 2006; Wahlander*, et al.*, 2006; Zafar*, et al.*, 2007; Zafar*, et al.*, 2007] but has not been used to assess fibrinolysis.

By combining two well-characterised and established techniques, the aim of the present study was to assess the ability of exogenous and endogenous t-PA to limit thrombus formation in a model of deep coronary arterial injury.

4.3 METHODS

4.3.1 SUBJECTS

Twenty healthy non-smokers aged between 18 and 26 years were enrolled into the study that was performed with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and the written informed consent of all volunteers. Exclusion criteria were bleeding diathesis, the use of regular medication or any clinically significant illness. Except for study medication, none of the subjects received vasoactive medication during the study and all abstained from alcohol for 24 hours and from food and caffeine-containing drinks for at least six hours before each study. Studies were carried out with subjects lying supine in a quiet, temperature-controlled room maintained between 22°C and 25°C.

4.3.2 *EX VIVO* THROMBOSIS STUDIES

Thrombus formation was measured using the Badimon Chamber as described in section 2.3.

Histological Analysis

As thrombus forms along the entire length of the exposed porcine aortic strip, the cross-sectional area gives a reliable reflection of total thrombus [Dangas*, et al.*, 1998]. Following fixation, the proximal and distal 1-mm sections of the exposed substrate were discarded and the remainder cut into four pieces. These pieces were paraffin-wax embedded and 5 μm sections were prepared from each in order that quantification was performed on sections taken from all parts of the exposed tissue strip.

Sections were stained with Masson's trichrome stain to detect total thrombus or with an anti-fibrin II β chain mouse monoclonal antibody (clone T2G1; Accurate Chemical & Scientific Corporation, USA) to detect fibrin. For immunohistochemical staining, endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide and serum free protein block (Dako, Glostrup, Denmark). Sections were then incubated with the primary antibody at a final concentration of 10 μg/mL for 1 hour at room temperature. Detection was performed using EnVision™ (Dako, Denmark) and treatment with 3,3'-diaminobenzidine substrate chromogen (Dako, Denmark). Finally, sections were counterstained with haematoxylin. No staining was present on specimens not incubated with the primary antibody or on areas of the specimens not exposed to blood flow.

A purpose-designed semi-automated scanning microscope and image analysis system (Ariol 3.1, Applied Imaging, USA) was used to quantify thrombus area and composition. Digital images of each section were acquired at ×20 magnification. High-resolution classifiers, based on colour and shape parameters, were established to detect total thrombus and fibrin staining. Results from at least six sections were averaged to determine thrombus area for each chamber as described previously [Lev*, et al.*, 2006; Wahlander*, et al.*, 2006; Zafar*, et al.*, 2007].

4.3.3 FOREARM BLOOD FLOW

To confirm intrarterial infusion of the vasoactive agents, blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silicone elastomer strain gauges as previously described [Webb, 1995]. Heart rate and blood pressure were monitored in the non-infused arm throughout each study with a non-

invasive, semi-automated oscillometric sphygmomanometer (Boso Medicus, Germany).

4.3.4 BLOOD SAMPLING AND FIBRINOLYTIC ASSAYS

Blood samples were taken immediately distal to the perfusion chambers (and from the non-infused arm where appropriate) during each study and collected into citrate for PAI-1 assay and acidified buffered citrate (Stabilyte^{TM}, Biopool International, USA) for t–PA and D-dimer assays. Samples were kept on ice before being centrifuged at 2000 *g* for 30 min at 4 °C. Platelet-free plasma was decanted and stored at -80 °C prior to assay. Plasma t-PA antigen and activity (t-PA Combi Actibind Elisa Kit, Technoclone, Austria), PAI-1 antigen (Zymutest, Hyphen Biomed, France) and D-dimer (Asserachrom® D-dimer, Roche, Switzerland) concentrations were determined using enzyme-linked immunosorbent assays.

4.3.4 STUDY DESIGN

Exogenous t-PA Administration

To assess the effect of recombinant t-PA (rt-PA) administered into the extracorporeal circuit, eight subjects attended on two separate occasions, two weeks apart in a double-blind randomised crossover design. On each visit, two *ex vivo* thrombosis studies were performed 45 min apart during extracorporeal administration of rt-PA (Boehringer Ingelheim, Germany; infused to achieve anticipated final concentrations of 40, 200 and 1000 ng/mL) or saline control. Study drugs were added to the extracorporeal circuit using a calibrated syringe driver (Alaris Arsena GH, Cardinal Health, USA) and allowed to mix prior to entering the perfusion chambers as previously described [Lucking*, et al.*, 2010]. Samples for fibrinolytic components

were taken from the chamber effluent and thrombus formation assessed by measuring fibrin and total thrombus area.

Endogenous t-PA Release

We assessed the ability of endogenous t-PA released from the vascular endothelium to limit acute thrombus formation in a separate cohort of 12 subjects who attended on two separate occasions, two weeks apart, having received matched placebo or the angiotensin-converting enzyme (ACE) inhibitor, enalapril, 10 mg once daily for seven days prior to attendance in a double-blind randomised crossover design. On each study day, the final dose of placebo or enalapril was taken at 08.00 hr. Four hours later, the subjects rested recumbent, and strain gauges and cuffs were applied to the forearms. The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd., Birmingham, UK) under local anaesthesia (1% lidocaine). The total rate of intra-arterial infusions was maintained constant at 1 mL/min. Forearm blood flow was measured every 3 min during infusion of vasodilators and every 5 min during infusion of saline. Intrabrachial infusions of bradykinin (MSD, UK; endothelium-dependent vasodilator releasing t-PA) at 1,000 pmol/min [Labinjoh*, et al.*, 2001; Witherow*, et al.*, 2002] and sodium nitroprusside (David Bull Laboratories, UK; endothelium independent vasodilator not releasing t-PA) at 8 μg/min [Labinjoh*, et al.*, 2000; Newby*, et al.*, 1997] were given for 12 min in a randomised order. Saline was infused for 30 min between vasodilator infusions. Samples for fibrinolytic components were taken from the chamber effluent and the non-infused arm. Total thrombus area was assessed at baseline (i.e. during intrabrachial infusion of saline) and during infusion of bradykinin and sodium nitroprusside.

4.3.5 DATA ANALYSIS AND STATISTICS

The sample size was selected based on previous studies suggesting that we required a sample size of n=8-12 to have an 80% power to detect differences of 6-10% in thrombus area at $p<0.05$ [Fernandez-Ortiz*, et al.*, 1994; Lucking*, et al.*, 2008; Osende*, et al.*, 2001; Toschi*, et al.*, 1997].

Plethysmographic data were extracted from the Chart data files. Forearm blood flows were calculated for individual venous occlusion cuff inflations by a template spreadsheet (Excel v12.3; Microsoft Corp., USA) as previously described [Labinjoh*, et al.*, 2000; Newby*, et al.*, 1999; Newby*, et al.*, 1997].

Continuous variables are reported as mean ± standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism (GraphPad Software, USA) using paired Student's *t*-tests and analysis of variance (ANOVA) as appropriate. Statistical significance was taken at $P<0.05$.

4.4 RESULTS

All study visits were completed for each subject without significant adverse events.

4.4.1 EXTRACORPOREAL RECOMBINANT T-PA

Plasma t–PA antigen concentrations in the venous effluent of the chamber (6.0±0.6, 44±6.6, 264±13 and 1567±177 ng/mL) approximated to the anticipated plasma t–PA concentrations (5, 40, 200 and 1000 ng/mL respectively; Figure 4.1). There was a dose-dependent increase in plasma D-dimer concentrations ($p<0.0001$; Figure 4.1) accompanied by a corresponding dose-dependent reduction in total and fibrin-rich

thrombus under both low and high shear conditions ($p<0.001$ for all; Figure 4.2). Thrombus in the low shear chamber had a greater proportion of fibrin compared to the high shear chamber (23±5% *versus* 8±2%, $p<0.001$; Figure 4.2).

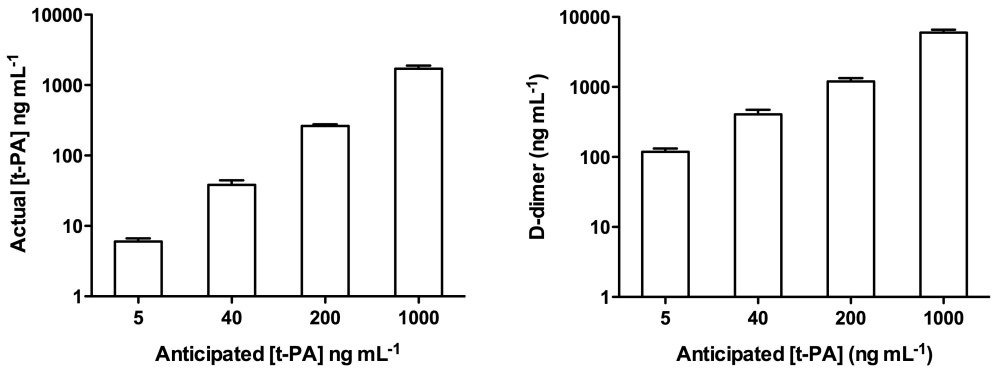

Figure 4.1 *The effect of recombinant tissue plasminogen activator administered into the extracorporeal circuit of the Badimon Chamber on plasma fibrinolytic components measured in the chamber effluent (n=8). Data shown are mean ± standard error of the mean.*

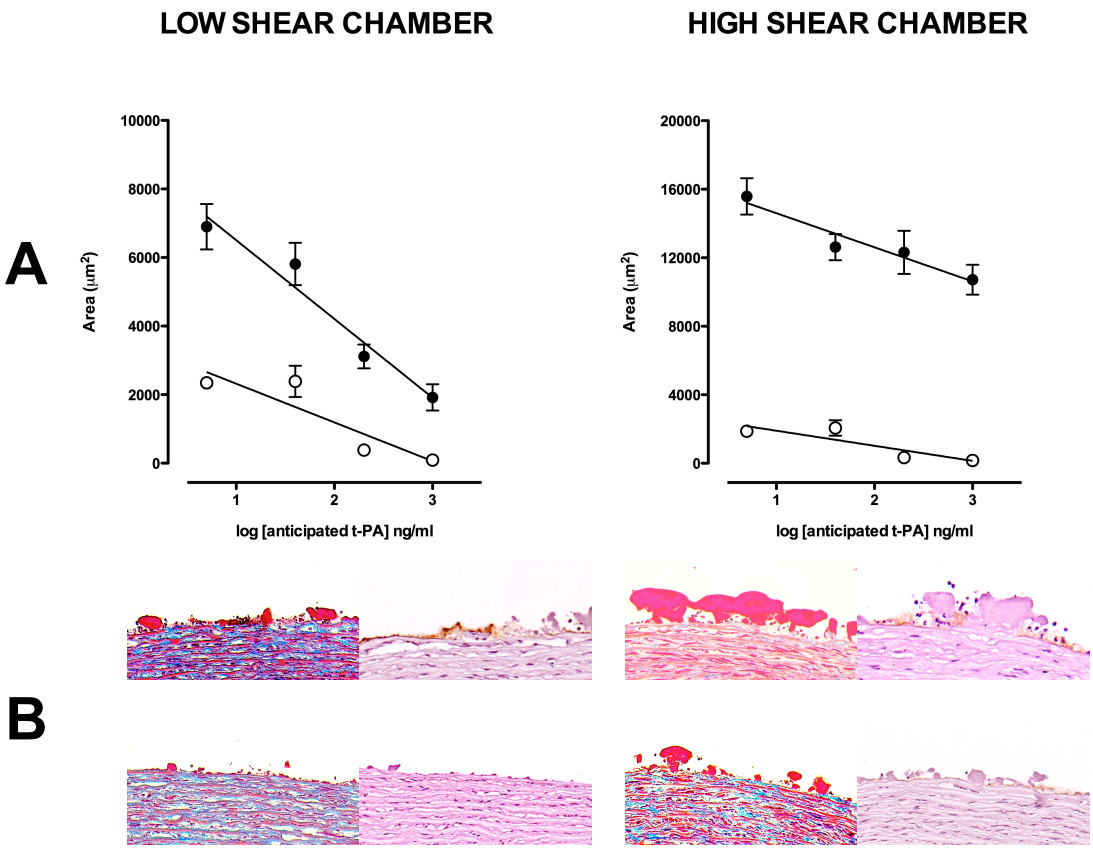

Figure 4.2. (A) *The effect of recombinant tissue plasminogen activator (rt-PA) administered into the extracorporeal circuit of the Badimon Chamber on total thrombus area (closed circles) and fibrin-rich thrombus area (open circles)(n=8). Data shown are mean ± standard error of the mean.* **(B)** *Representative photomicrographs of porcine aortic media exposed to flowing blood demonstrating the effect of rt-PA administered into the extracorporeal circuit of the Badimon Chamber. Photomicrographs in the upper panels are from studies with the addition of saline control and those in the lower panels from studies with the addition of rt-PA 1000 ng/mL. Sections on the left of each panel are stained with Masson's trichrome stain to demonstrate total thrombus area. Sections on the right are stained for fibrin and counterstained with haematoxylin to demonstrate fibrin-rich thrombus area (fibrin appears brown). Original magnification × 20.*

4.4.2 ENDOGENOUS FIBRINOLYSIS

Oral and intra-arterial drug administrations were well tolerated without significant adverse effects. Consistent with previous studies [Labinjoh*, et al.*, 2000], transient patchy flushing and skin oedema of the infused arm occurred with bradykinin infusion. There were no changes in heart rate, blood pressure and non-infused forearm

blood flow during or between study days (data not shown). As previously, sodium nitroprusside and bradykinin produced dose-dependent forearm vasodilatation ($p<0.001$ for all; data not shown).

Compared to saline, sodium nitroprusside had no effect on fibrinolytic parameters in the presence of placebo or enalapril (data not shown). Intra-arterial bradykinin increased plasma t-PA antigen and activity in the chamber effluent during treatment with placebo and enalapril ($p<0.01$ for all *versus* saline; Figure 4.3). Bradykinin-induced t-PA antigen concentrations were increased approximately four-fold and t-PA activity increased approximately seven-fold in the presence of enalapril ($p<0.0001$ for both *versus* placebo; Figure 4.3) such that systemic spillover and elevation of plasma t-PA antigen and activity in the non-infused arm were observed ($p=0.01$ and $p<0.001$ respectively *versus* saline; Figure 4.3). These changes in plasma t-PA antigen and activity were accompanied by an increase in plasma D-dimer concentrations in the chamber effluent during placebo and enalapril treatment ($p=0.003$ and $p=0.001$ respectively *versus* saline; Figure 4.3), confirming that t-PA released from the forearm endothelium enhanced fibrinolysis within the chamber. PAI-1 antigen concentrations were unaffected by the presence of enalapril or during intra-arterial administration of bradykinin (Figure 4.3).

The changes in fibrinolytic markers were accompanied by a reduction in total thrombus area in the low shear chamber during bradykinin infusion (15±5%, $p=0.026$ *versus* saline) and a trend towards a reduction in the high shear chamber (13±7%, $p=0.07$ *versus* saline; Figure 4.4). There were no differences in thrombus area in either the low or high shear chambers in the presence of placebo (Figure 4.4).

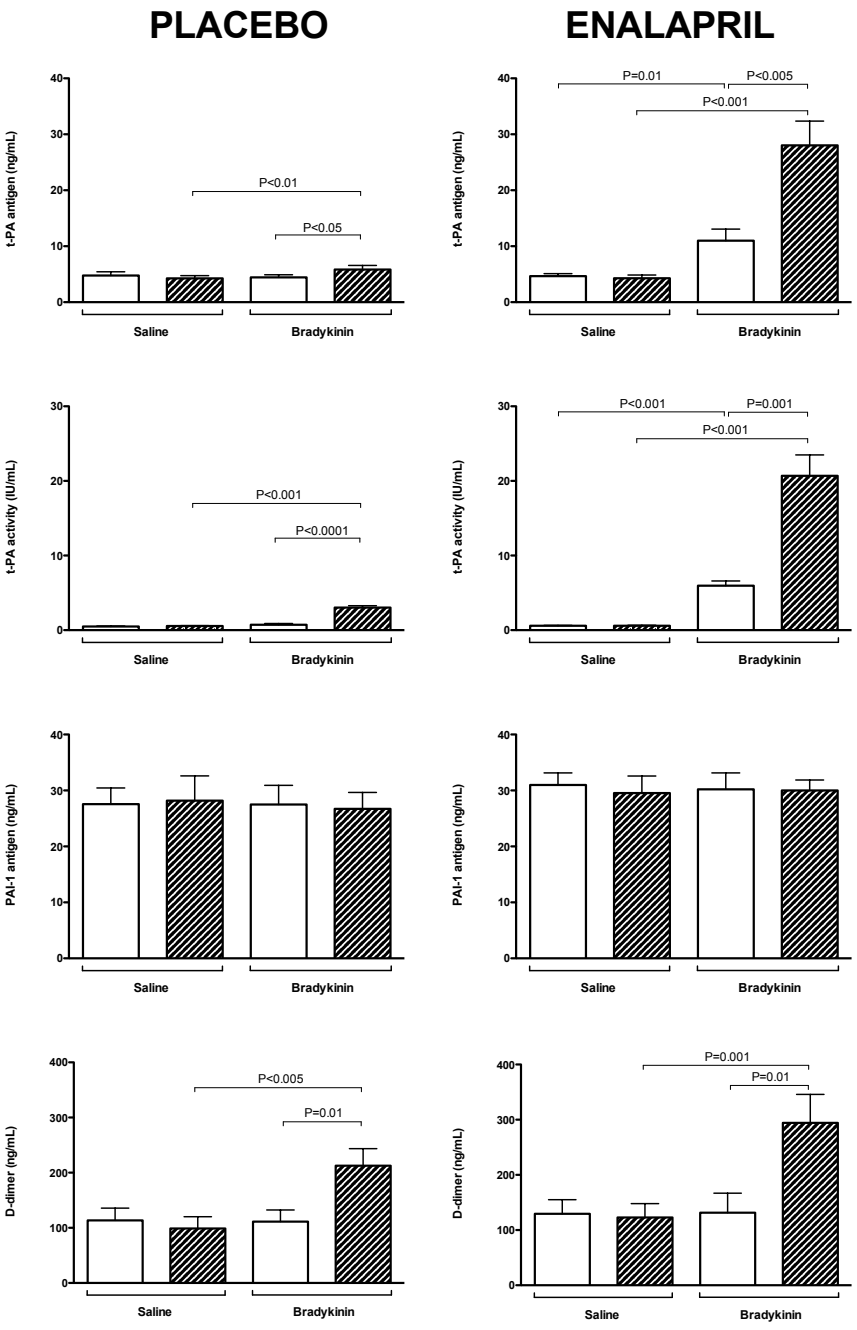

Figure 4.3. *The effect of intra-arterial bradykinin infusion on plasma fibrinolytic components measured in the non-infused arm (clear bars) and the chamber effluent (hashed bars)(n=12). Data shown are mean ± standard error of the mean.*

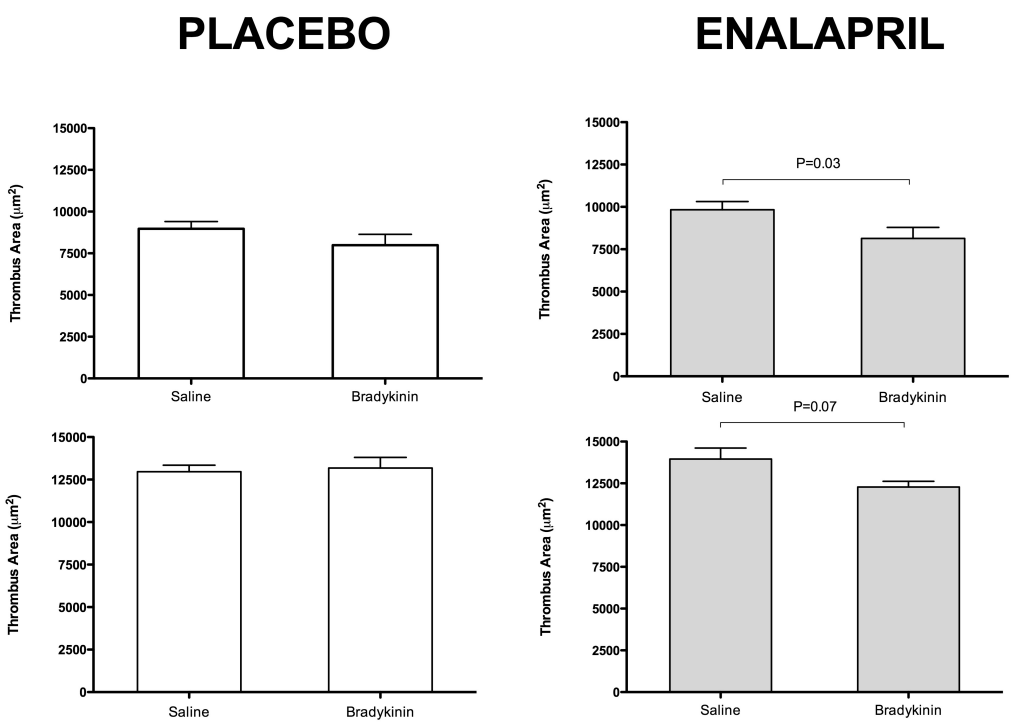

Figure 4.4. *The effect of intra-arterial bradykinin infusion on total thrombus area assessed in the Badimon Chamber in the presence of placebo (clear bars) and the angiotensin-converting enzyme inhibitor, enalapril (filled bars) (n=12). Upper panels show data from the low shear chamber, lower panels show data from the high shear chamber. Data shown are mean ± standard error of the mean.*

4.5 DISCUSSION

Recent work has provided novel insights into the role of endogenous fibrinolysis. Using intra-arterial infusion in the human forearm and coronary circulations, we [Chia*, et al.*, 2003; Gudmundsdottir*, et al.*, 2006; Labinjoh*, et al.*, 2000; Labinjoh*, et al.*, 2001; Newby*, et al.*, 2001; Newby*, et al.*, 2002; Newby*, et al.*, 1997; Witherow*, et al.*, 2002] and others [Brown*, et al.*, 2000; Brown*, et al.*, 1999; Hrafnkelsdottir*, et al.*, 2004; Hrafnkelsdottir*, et al.*, 1998; Jern*, et al.*, 1994] have demonstrated that a variety of agonists can stimulate t-PA release from the human vascular endothelium *in vivo*. In the current study, we have confirmed that acutely released endogenous endothelial t-PA is functionally active, enhances local fibrinolysis and can limit *in situ* thrombus formation. This establishes the clinical significance of this method of assessing endothelial function as well as underscoring the importance of this aspect of vascular function.

The endothelium has a number of important complementary functions, including regulation of vasomotor tone, coagulation, fibrinolysis and inflammation. Most clinical studies have focused on endothelium-dependent vasomotion. Whilst a useful surrogate marker for the role of the endothelium in atherothrombosis [Perticone*, et al.*, 2001] the observation of impaired t-PA release with preservation of vasomotor function in response to endothelium-dependent vasodilators in cigarette smokers [Chowienczyk*, et al.*, 1993] and patients with hypertension [Hrafnkelsdottir*, et al.*, 2004; Hrafnkelsdottir*, et al.*, 1998] highlights both the complexity of vascular biology and suggests that fibrinolytic capacity may be a more sensitive marker of endothelial dysfunction in some circumstances.

Despite a growing body of evidence, a key limitation of studies to date is that the effects of acute endogenous t-PA release on *in situ* thrombus formation have not been demonstrated. Is endogenous endothelial t-PA released under agonist stimulation functionally active and able to enhance fibrinolysis of *in situ* thrombus? Clearly, it would be unethical to induce intra-arterial thrombus in the human forearm and there is currently no model for assessing thrombus formation *in vivo* in the human circulation. In the present study, we attempted to resolve these problems by combining two well-established techniques: the Badimon Chamber, an *ex vivo* clinical model of thrombus formation, and the forearm technique, using intrabrachial infusion of bradykinin to generate enhanced concentrations of endogenous t-PA from the vascular endothelium. This methodology provides an elegant means of stimulating local t-PA release from the endothelium before channelling the venous effluent of the forearm, containing freshly released endogenous t-PA, through the Badimon Chamber allowing an assessment of *ex vivo* thrombus formation to be made. We initially validated our model using the extracorporeal administration of exogenous recombinant t-PA prior to assessing the effects of endogenous t-PA. The doses of t-PA used in the initial studies were selected based on data from previous forearm studies and measurements of t-PA concentration made during studies of systemic thrombolysis for myocardial infarction. We estimated that 40 ng/mL was the maximum t-PA antigen concentration achievable in healthy volunteers during bradykinin infusion in the presence of ACE inhibition [Labinjoh*, et al.*, 2001; Witherow*, et al.*, 2002]. Concentrations of 200 ng/mL and 1000 ng/mL were selected to represent the lower and upper ends respectively of plasma t-PA antigen concentrations achieved during systemic therapeutic fibrinolysis [Genser*, et al.*, 1998; Koster*, et al.*, 1991]. Based on this rationale, we were able to demonstrate that across a clinically relevant dose range,

thrombus formation was reduced in a dose-dependent manner. Consistent with its mechanism of action, exogenous t-PA was associated with reductions in fibrin-rich clot and an elevation in D-dimer concentrations in the venous effluent.

Having validated our model, we then wanted to explore whether freshly released endogenous t-PA would have similar effects to exogenous t-PA. We employed both systemic ACE inhibition and intra-arterial bradykinin administration to maximise endogenous t-PA release. Using this approach, we were able to increase plasma t-PA concentrations to those seen with the lowest dose of exogenous t-PA. Consistent with this, we observed comparable reductions in thrombus area and increases in D-dimer formation. These data therefore confirm the importance of the endogenous fibrinolytic system and the use of the forearm model as a relevant model with which to assess acute endogenous fibrinolytic capacity. Furthermore, we believe that this methodology provides a robust, versatile and relevant means with which to assess novel agents with the potential to modulate the endogenous fibrinolytic system and thus may benefit patients with a broad range of disease processes.

We elected to use bradykinin as the agonist to stimulate t-PA release for two principal reasons. Firstly, it is pathophysiologically relevant. Although an inflammatory mediator, it is also released during the contact phase of coagulation and is a potent endothelial cell activator, stimulating release of t-PA from the endothelium [Brown*, et al.*, 1999; Labinjoh*, et al.*, 2000] through a B2 receptor mediated mechanism [Brown*, et al.*, 2000]. Thus, following activation of the intrinsic coagulation pathway, the liberation of bradykinin may represent an important negative feedback loop in which bradykinin-induced t-PA release inhibits thrombus formation within the vascular

lumen when localised endothelial denudation occurs. Secondly, the use of bradykinin affords a safe and efficacious means of maximising t-PA release. Ordinarily, bradykinin has a very short plasma half-life of ~15 seconds with >95% of its metabolism occurring through ACE. In the presence of ACE inhibition, bradykinin-induced vasodilatation and t-PA release are selectively and markedly potentiated [Benjamin*, et al.*, 1989; Labinjoh*, et al.*, 2001; Witherow*, et al.*, 2002] and this may account for some of the anti-ischemic actions of ACE inhibition in vascular disease [Yusuf*, et al.*, 2000]). Accordingly, in the presence of placebo, infusion of bradykinin resulted in more modest increases in plasma t-PA antigen and activity in the infused arm, sufficient to result in an increase in plasma D-dimer concentration confirming enhanced fibrinolysis but not sufficient enough to detect a significant change in thrombus formation in the Badimon Chamber. However in the presence of ACE inhibition, there was a roughly four-fold increase in t-PA antigen and seven-fold increase in t-PA activity sufficient to result in systemic spillover and a marked elevation of plasma t-PA antigen and activity in the *non-infused* arm. These marked increases in local and systemic plasma t-PA antigen and activity are consistent with our previous studies [Labinjoh*, et al.*, 2001; Witherow*, et al.*, 2002] and resulted in an increase in plasma D-dimer concentrations and an accompanying reduction in thrombus formation. In contrast to a previous study in patients with moderate to severe heart failure [Witherow*, et al.*, 2002], basal plasma PAI-1 antigen concentrations were not affected by ACE inhibition, suggesting that in this healthy volunteer cohort, enhanced endogenous fibrinolysis was due to an increase in t-PA activity rather than a reduction in PAI-1 antigen concentration.

Although the magnitude of reduction in thrombus area with endogenous t-PA release

was relatively modest and seen only in the presence of ACE inhibition, this occurred despite the marked dilutional effect that occurs because of the substantial increase in blood flow associated with concomitant bradykinin-induced vasodilatation. At the site of unstable plaques within the coronary circulation, it is likely that local concentrations will be much higher and, with impending vessel occlusion, dilutional effects will be minimised. Furthermore, we studied healthy volunteers with intact vasomotor responses and no vascular dysfunction. In our previous studies, ACE inhibition had a disproportionately large effect on bradykinin-induced t-PA release in patients with established heart disease [Witherow*, et al.*, 2002]. Indeed, in these patients, we were able to demonstrate up to 4.5 µg of endogenous t-PA release from the forearm and achieve 99 IU/mL of t-PA activity that approached the activity seen with systemic therapeutic fibrinolysis (100-1000 IU/mL) [Witherow*, et al.*, 2002]. This may, in part, be due to impaired vasodilatation causing the accumulation of greater plasma concentrations of released t-PA. Taken together, these considerations suggest that local t-PA concentrations generated in the coronary circulation *in vivo*, particularly in patients treated with ACE inhibition, may be higher than those we achieved here. It does however remain possible that bradykinin and/or enalapril are affecting aspects of platelet function, coagulation or the fibrinolytic system that we have not measured here.

4.5.1 STUDY LIMITATIONS

We acknowledge the modest sample size and exclusive use of healthy volunteers. It would be desirable to increase subject numbers and make similar assessments in patients with vascular disease but the invasive nature and complexity of combining two exacting techniques presents many challenges particularly when attempting to

apply this methodology to larger cohorts and patient groups.

Measurement of coronary t-PA release is of greatest relevance to coronary pathophysiology but can only be performed in selected subjects undergoing invasive coronary angiography. However, consistent findings between the forearm [Labinjoh*, et al.*, 2001; Newby*, et al.*, 1999; Witherow*, et al.*, 2002] and coronary circulations [Minai*, et al.*, 2001; Newby*, et al.*, 2001] support the notion that the use of the forearm model is a reasonable surrogate, particularly in the context of this proof-of-concept study.

For the reasons discussed earlier, we believe that our rationale for choosing bradykinin as the agonist with which to stimulate t-PA release is sound, although we acknowledge that the local concentration generated at the surface of an unstable atherosclerotic plaque and the exact role played by bradykinin in potentiating the endogenous fibrinolytic system *in vivo* remains incompletely understood.

4.5.2 CONCLUSIONS

Using a well-characterised clinical model of arterial injury, we have demonstrated that endogenous t-PA released acutely from the human vascular endothelium enhances fibrinolysis and limits *in situ* thrombus formation. These data confirm the functional significance of t-PA released during agonist stimulation, support a crucial role for the endogenous fibrinolytic system and suggest that further studies to explore its therapeutic value are warranted.

CHAPTER 5

EFFECT OF THE SMALL MOLECULE PLASMINOGEN ACTIVATOR INHIBITOR-1 (PAI-1), PAI-749, IN CLINICAL MODELS OF FIBRINOLYSIS

Published by **Lucking AJ**, Visvanathan A, Philippou H, Fraser S, Grant PJ, Connolly TM, Gardell SJ, Feuerstein GZ, Fox KA, Booth NA, Newby DE.
J Thromb Haemost. 2010 Jun; 8(6):1333-9.

5.1 SUMMARY

The principal inhibitor of fibrinolysis *in vivo* is plasminogen activator inhibitor type 1 (PAI–1). PAI–749 is a small molecule inhibitor of PAI–1 with proven antithrombotic efficacy in several pre-clinical models. The aim of this study was to assess the effect of PAI–749 using an established *ex vivo* clinical model of thrombosis and a range of complementary *in vitro* human plasma and whole blood-based models of fibrinolysis. In a double-blind randomised crossover study, *ex vivo* thrombus formation was assessed using the Badimon Chamber in 12 healthy volunteers during extracorporeal administration of t–PA in the presence of PAI–749 or control. Tissue plasminogen activator (t–PA) mediated lysis of plasma clots and whole blood model thrombi were assessed *in vitro*. The role of vitronectin was examined by assessing lysis of fibrin clots generated from purified plasma proteins. There was a dose-dependent reduction in *ex vivo* thrombus formation by t–PA ($P<0.0001$). PAI–749 had no effect on *in vitro* or *ex vivo* thrombus formation or fibrinolysis in the presence or absence of t–PA. Inhibition of PAI–1 with a blocking antibody enhanced fibrinolysis *in vitro* ($P<0.05$).

Despite efficacy in a purified human system and in pre-clinical models of thrombosis, the current study suggests that PAI–749 does not affect thrombus formation or fibrinolysis in a range of established human plasma and whole blood-based systems.

5.2 INTRODUCTION

The release of tissue plasminogen activator (t–PA) from vascular endothelial cells initiates the dissolution of intravascular thrombus and is of particular importance within the coronary circulation [Rosenberg*, et al.*, 1999]. Indeed, exogenous administration of t–PA remains an important treatment for ST segment elevation myocardial infarction.

Plasminogen activator inhibitor type 1 (PAI–1) is a serine protease inhibitor that blocks the action of t–PA. Along with α_2-antiplasmin, PAI–1 is a key inhibitor of fibrinolysis *in vivo* [Robbie*, et al.*, 1996]. Elevated plasma PAI–1 concentrations are associated with a number of prothrombotic conditions including acute coronary syndrome [Juhan-Vague*, et al.*, 1996], metabolic syndrome [Alessi*, et al.*, 2006] and venous thromboembolism [Crowther*, et al.*, 2001].

Inhibition of PAI–1 is predicted to promote endogenous and exogenous t–PA mediated fibrinolysis. This may enhance the therapeutic efficacy of thrombolytic agents as well as having the potential to prevent adverse cardiovascular events through augmentation of endogenous fibrinolysis. PAI–749 is a novel synthetic small molecule inhibitor of PAI–1 that has advanced to clinical phase I evaluation. It has been shown to inhibit PAI–1 rapidly and selectively *in vitro* [Gardell*, et al.*, 2007]. In rodent and canine models, PAI–749 limits *de novo* thrombus formation and accelerates lysis of pre-existing thrombi in both venous and arterial systems [J. Hennan, 2006].

The principal aim of this study was to assess the effect of PAI–749 on t–PA mediated fibrinolysis using an established *ex vivo* clinical model of thrombosis. In addition, the effect of PAI–749 was assessed using a range of complementary *in vitro* human plasma and whole blood-based models of fibrinolysis.

5.3 METHODS

5.3.1 PAI–749

PAI–749, 1-benzyl-3-pentyl-2-[6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-1H-indolemin (MW 502 Da), was synthesised and characterised for chemical identity and purity at Wyeth Research (Collegeville, USA). Aliquots of stock solution (0.5 mg/mL; 1 mM) were prepared in 50% PEG-200/50% sterile water and stored at -80°C prior to use. A single batch of PAI–749 was used for all studies.

The ability of PAI–749 to inhibit PAI–1 was confirmed by use of a chromogenic assay[Gardell*, et al.*, 2007] and in our hands the IC50 of PAI–749 was 295 nM. The PAI–1 used was recombinant human PAI–1, the specific activity of which was approximately 35%.[Sancho*, et al.*, 1994]

5.3.2 *IN VITRO* STUDIES

Plasma Clot Lysis

The effect of PAI–749 on clot lysis was studied in a 30% plasma system using thrombin/Ca^{2+} to induce clot formation and t–PA to induce clot lysis, as described previously [Mutch*, et al.*, 2007]. Platelet-rich or platelet-free plasma was mixed with t–PA and either antibody to PAI–1 or PAI–749 in a final volume of 200 μL 10 mM Tris pH 7.5, 0.01% (v/v) Tween 20 (Tris-Tween buffer). Aliquots from each tube (80 μL) were added in duplicate to microtitre wells, each containing 20 μL thrombin/$CaCl_2$ to give the following final concentrations per well: 30% (v/v) plasma, 0.4 IU/ml thrombin, 10.6 mM $CaCl_2$, 180 pM t–PA, with or without 200 μg/mL rabbit polyclonal antibody to human PAI–1 [Booth*, et al.*, 1987; Booth*, et al.*, 1988] or PAI–749 (0, 2.5 or 12.5 μg/mL; 0, 5 or 25 μM). The plate was incubated at 37°C and

read at 405 nm at 5 min intervals [Mutch*, et al.*, 2007].

Lysis of Whole Blood Model Thrombi Formed Within a Chandler Loop

FITC-labelled fibrinogen was added to citrated whole blood (0.9 mL) taken from healthy donors [Mutch*, et al.*, 2007]. Samples were recalcified with 10.9 mM $CaCl_2$ in a volume of 1.15 mL. The blood was then placed in plastic tubing and rotated at 30 rpm for 90 min as described previously [Mutch*, et al.*, 2007]. Once formed, model thrombi were rinsed in 0.9% (v/v) saline, blotted with filter paper and transferred to a tube containing t–PA (final concentration 200 ng/mL) in 10 mM Tris buffer, 0.01% (v/v) Tween, pH 7.5. PAI–749 (final concentrations 0.5, 2.5, or 12.5 mg/mL; 1, 5 or 25 μM) or anti-PAI–1 antibody (200 μg/mL) were incorporated in the thrombi and/or added to bathing fluid. Samples were removed at intervals during incubation at 37 °C and the released fluorescence was measured in a fluorescence plate reader at excitation, 485 nm and emission, 530 nm [Mutch*, et al.*, 2007].

Lysis of Fibrin Clots Formed Within a Chandler Loop

Purified proteins were used to generate fibrin clots under flow conditions within a Chandler Loop in a variation of the technique described above [Mutch*, et al.*, 2003]. Time to complete lysis was monitored visually without wash steps. Clots were generated using final concentrations of 0.5 mg/mL of fibrinogen (Calbiochem, UK), 0.11 IU/mL of thrombin (Calbiochem, UK), 100 ng/mL of t–PA (Technoclone, Austria), 66.6 μg/mL of plasminogen (Enzyme Research Laboratories, UK) in 50 mM Tris buffer with 100 mM NaCl, 7.5 mM $CaCl_2$, pH 7.4. Thrombin, t–PA and calcium were added to the reagent mixtures as a single mix to initiate clot formation. Samples were then placed in plastic tubing and rotated at 30 rpm with constant visual

monitoring. Clots formed within 5 min (reaching a maximal size between 15-20 min) and the time taken for each to disappear completely was noted. Clots were prepared using the reagents listed above with the addition of recombinant human PAI–1 [Sancho*, et al.*, 1994] (final concentration 408 ng/mL) ± PAI–749 (final concentration 2.5 μg/mL; 5 μM) ± vitronectin (Stratech Scientific, UK; final concentration 300 μg/mL).

5.3.3 *EX VIVO* STUDIES

Subjects

Twelve healthy non-smokers aged between 21 and 28 years old were recruited. The study was conducted with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and with the written informed consent of all volunteers. Exclusion criteria were the use of regular medication or any clinically significant illness.

Study Design

Subjects attended on three occasions in a double-blind randomised crossover design. *Ex vivo* thrombus was generated in the Badimon chamber. The effect of extracorporeal administration of t–PA (Boehringer Ingelheim, Germany; infused to achieve estimated final concentrations of 5, 40 and 200 ng/mL) was assessed in the presence of PAI–749 (Wyeth Research, USA; targeted final concentration 2.5 μg/mL), tranexamic acid (Meda Pharmaceuticals, UK; targeted final concentration 0.5 μg/mL) or saline control.

Thrombosis Studies

Thrombus formation was measured using the Badimon Chamber as described in section 2.3.

Immediately after each study, the porcine strips with thrombus attached were removed and fixed in 4% (w/v) paraformaldehyde for 72 h. Strips were then paraffin-wax embedded, sectioned and stained with Masson's Trichrome. Digital images were acquired at ×20 magnification, and thrombus area measured using a semi-automated image acquisition system (Ariol, Applied Imaging, USA) by a second blinded operator. Results from at least six sections were averaged to determine thrombus area for each chamber as described previously [Lucking*, et al.*, 2008].

Blood Sampling

Blood samples were taken immediately distal to the perfusion chambers during each study and collected into acidified buffered citrate (Stabilyte^{TM}, Biopool International, USA) for t–PA, PAI–1, t–PA/PAI–1 complex, D-dimer, and thrombin-antithrombin complex (TAT) assays, and ethylenediaminetetraacetic acid (BD Vacutainer, USA) for PAI–749 measurement. Samples for t–PA and PAI–1 assays were kept on ice before being centrifuged at 2000 *g* for 30 min at 4 °C. Samples for PAI–749 assay were kept at room temperature before being centrifuged at 1500 *g* for 30 min at 20 °C. Platelet-free plasma was decanted and stored at -80 °C prior to assay. D-dimer (Asserachrom® D-dimer, Roche, Switzerland) and TAT complex (Dade Behring, UK) concentrations were determined using enzyme-linked immunosorbent assays (ELISA). Plasma t–PA antigen [Booth*, et al.*, 1987], PAI–1 antigen and activity [MacGregor*, et al.*, 1988], and t–PA/PAI–1 [Bennett*, et al.*, 1990] complex were

determined as described previously. To determine plasma PAI–749 concentrations in the chamber effluent, 100 μL of calibration standard, quality control sample, blank, zero standard, or study sample were mixed with 50 μL of internal standard (D_{11}-PAI–749 [compound isotope to facilitate detection by mass spectroscopy], 500 ng/mL in methanol/5 mM ammonium acetate, v/v). The mixture was precipitated with 0.6 mL of acetonitrile and 0.3 mL of the supernatant mixed with 0.1 mL of 5 mM ammonium acetate. The processed samples were then analysed using LC/MS/MS.

5.3.4 STATISTICAL ANALYSIS

Continuous variables are reported as mean ± standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism (GraphPad Software, USA) by two-way analysis of variance (ANOVA) with repeated measures, paired Student *t* tests and calculation of the Pearson correlation coefficient as appropriate. Statistical significance was taken at $P<0.05$.

5.4 RESULTS

5.4.1 *IN VITRO* STUDIES

Tissue plasminogen activator mediated lysis of platelet-poor and platelet-rich plasma clots was unaffected by PAI–749 (2.5 or 12.5 μg/mL; 5 or 25 μM) while the blocking antibody to PAI–1 caused an increase in lysis ($P<0.05$ for time points between 15 and 85 min; data not shown).

PAI–749 had no effect on t–PA mediated lysis of model thrombi formed in a Chandler loop at any of the concentrations tested. Indeed at 12.5 μg/mL, there was a decrease in lysis ($P<0.01$ for timepoints between 90 and 240 min with PAI–749 added to both blood and buffer; Figure 5.1A). The addition of antibody to PAI–1 resulted in enhanced lysis ($P<0.01$) between 120 and 240 min (Figure 5.1B).

PAI–749 had no effect on t–PA mediated lysis of fibrin clots formed from purified plasma proteins in a Chandler loop in the presence or absence of vitronectin (data not shown).

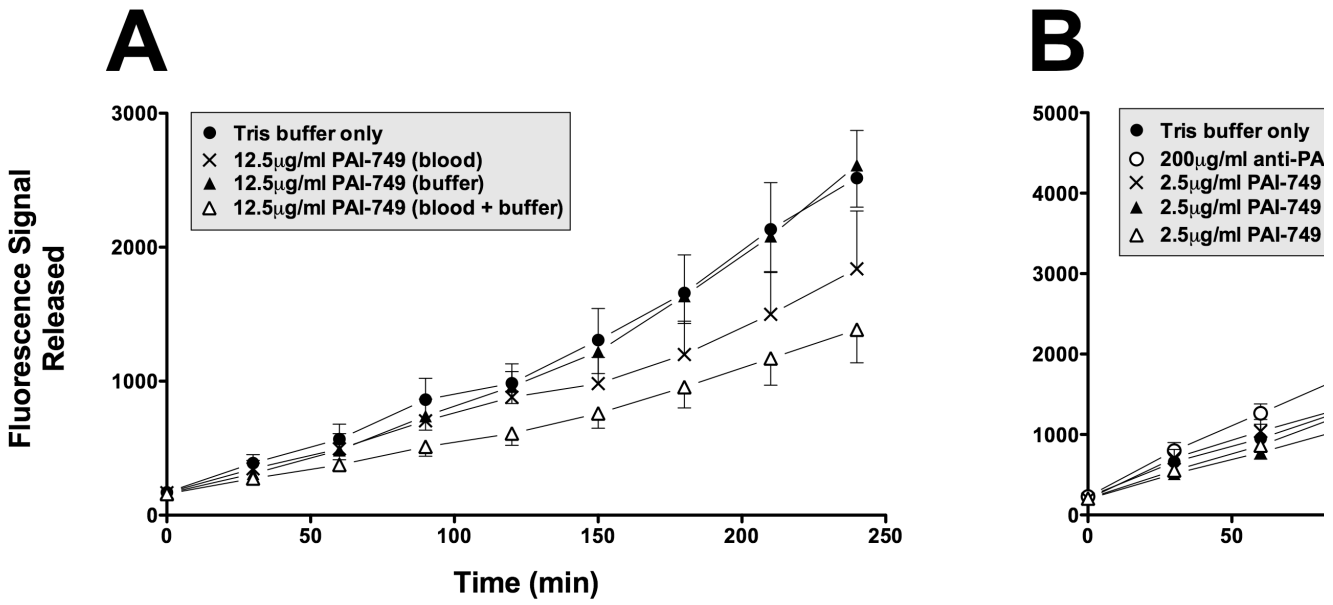

Figure 5.1 *Model thrombi were prepared in a Chandler loop from whole blood collected freshly from four volunteers. PAI–749 was added either to the blood prior to thrombus formation, to the bathing buffer during lysis or to both the blood and bathing buffer. Anti-PAI–1 neutralising antibody was added to both blood and bathing buffer. 200 ng/mL of tissue plasminogen activator was added to all bathing buffers. Lysis was monitored as fluorescence signal released due to the proteolysis of FITC-labelled fibrin. Data shown are mean±SEM.*

A. *The difference in lysis was not significant at any time point with PAI–749 added to the blood or bathing buffer alone. Lysis was decreased ($P<0.01$) between 90 and 240 min with 12.5 μg/mL PAI–749 in both blood and buffer.*

B. *There was no difference in lysis with added PAI–749. The addition of anti-PAI–1 resulted in an increase in lysis ($P<0.01$) between 120 and 240 min.*

5.4.2 *EX VIVO* STUDIES

Studies were completed in all 12 subjects and were well tolerated. Mean PAI–749 concentration was 3.3±0.1 μg/mL; similar to the maximum concentration achieved in phase I clinical studies (~2.5 μg/mL). PAI–749 was undetectable in control samples (data not shown).

Plasma Coagulation and Fibrinolytic Components

Plasma t–PA concentrations in the venous effluent of the chamber (3.9±1.5, 38±19 and 248±45 ng/mL respectively) closely matched the targeted t–PA concentrations (5, 40 and 200 ng/mL) during exogenous t–PA administration. There were no differences in plasma t–PA concentrations achieved during the three phases of the study: saline, PAI–749 (Table 5.1) or tranexamic acid (data not shown) infusion. Moreover, PAI–749 did not alter PAI–1 antigen, PAI–1 activity or t–PA/PAI–1 complex formation compared to saline control (P=0.76, 0.10 and 0.15, respectively; Table 5.1).

	SALINE			***PAI–749***		
Predicted t–PA (ng/ml)	**5**	**40**	**200**	**5**	**40**	**200**
Actual t–PA (ng/ml)	4.0±0.4	30±6	248±13	3.8±0.5	48±4	249±14
PAI–1 (ng/ml)	18±3	28±7	30±5	20±4	28±6	31±8
PAI activity (U/ml)	12±1	ND	ND	16±2	ND	ND
t–PA-PAI–1 complex (pM)	39±6	101±21	186±53	41±6	153±33	236±78
D-dimer (ng/ml)	231±65	388±71	994±177	130±19	603±206	637±136
TAT complex (ng/ml)	251±31	355±74	386±69	315±64	350±83	236±37

Data shown are mean ± SEM

ND - not determined

PAI–1 - plasminogen activator inhibitor type 1

TAT - thombin antithrombin complex

t–PA - tissue plasminogen activator

Table 5.1 *Plasma Fibrinolytic Components in the Badimon Chamber*

In keeping with *in situ* thrombus formation, TAT complexes were markedly elevated in all samples (274±21 μg/mL; normal range 1.0-4.1 μg/mL). Levels were unaffected by tranexamic acid or PAI–749 (P=0.79 and P=0.12 respectively). Consistent with a marked fibrinolytic effect, exogenous t–PA increased D-dimer concentrations in the venous effluent of the chamber (P<0.0001; Figure 5.2) in a dose-dependent manner

(r=0.67, P<0.0001). Plasma D-dimer concentrations were unaffected by tranexamic acid or PAI–749 (P=0.88).

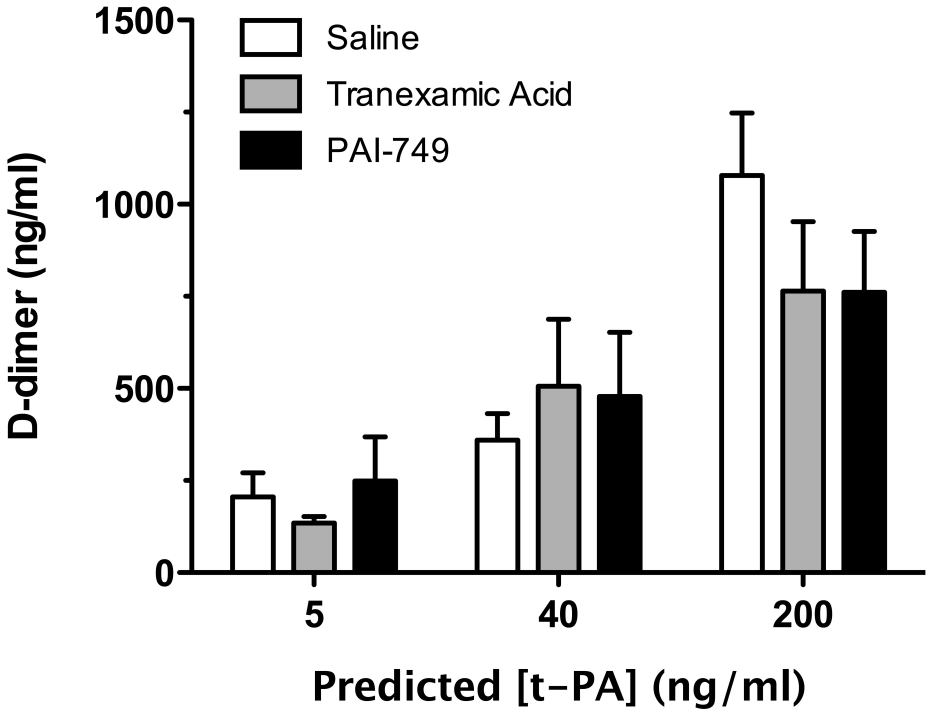

Figure 5.2 *D-dimer levels within the effluent of the Badimon chamber. Tissue plasminogen activator added to the extracorporeal circuit of the Badimon Chamber causes a dose-dependent increase in D-dimer concentration (P<0.0001) in the chamber effluent which is unaffected by tranexamic acid or PAI–749. (Data shown are mean ± SEM).*

Ex Vivo *Thrombus Formation*

Tissue plasminogen activator caused a dose-dependent reduction in thrombus formation in the Badimon chamber under low and high shear conditions (P<0.0001 for both; Figure 5.3). Whilst there appeared to be a trend towards thrombus stabilisation in the presence of tranexamic acid at the highest dose of t–PA (P=0.09

and P=0.06 *versus* saline in the low and high shear chambers respectively), PAI–749 had no effect on thrombus formation.

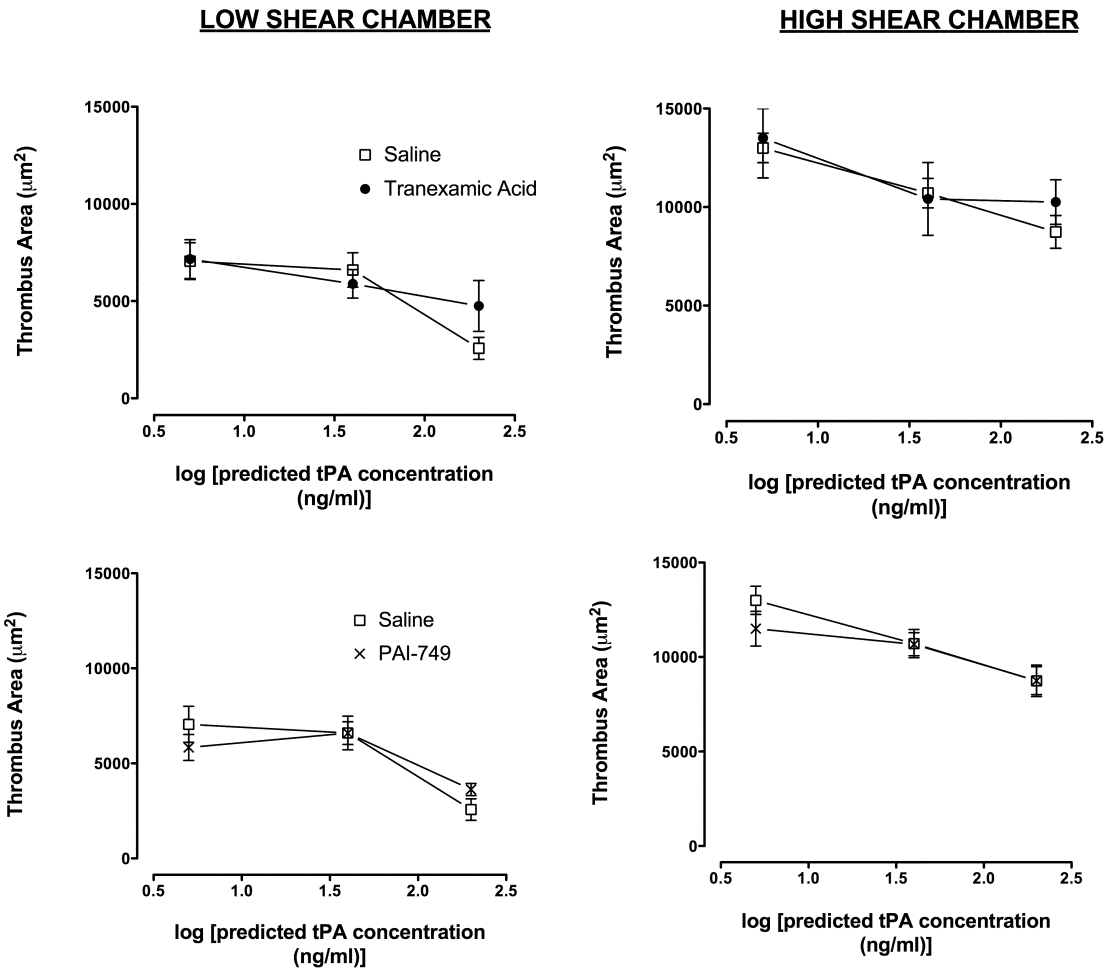

Figure 5.3. *Either tranexamic acid or PAI–749 along with tissue plasminogen activator were added to the extracorporeal circuit of the Badimon Chamber. Ex vivo thrombus formation was measured histomorphometrically. PAI–749 did not potentiate the action of baseline endogenous or exogenous tissue plasminogen activator). Data shown are mean ± SEM.*

Thrombus area correlated well with t–PA concentration in both the low and high shear chambers (r=-0.61, P<0.0001 and r=-0.49, P<0.0001 respectively). Thrombus

area also correlated with D-dimer concentration in the low shear chamber ($r=-0.52$, $P<0.0001$) and to a lesser extent in the high shear chamber ($r=-0.24$, $P=0.02$).

5.5 DISCUSSION

In the current studies, the novel small molecule PAI–1 inhibitor, PAI–749, did not potentiate the action of endogenous or exogenous t–PA on newly formed thrombus in clinical *in vitro* and *ex vivo* models. These findings are in contrast to the antithrombotic and profibrinolytic effects seen with PAI–749 in a variety of pre-clinical animal thrombosis models. The reason for the lack of efficacy in human blood needs to be established if the potential therapeutic strategy of PAI–1 inhibition is to be realised.

Work to date on the fibrinolytic system has principally been conducted using *in vitro* plasma-based systems. This has allowed the identification and characterisation of the key components of the fibrinolytic system. More recent studies have emphasised the importance of considering the overall balance of fibrinolysis in whole blood in which the contribution of cellular elements, such as platelets and leucocytes, can be assessed [Mutch*, et al.*, 2007].

In the present study, the effect of PAI–749 on t–PA mediated fibrinolysis in platelet-poor and platelet-rich plasma was first assessed using a turbidity-based system. The concentrations of PAI–749 used, 5 or 25 μM, were greatly in excess of the reported IC_{50} of 157 nM [Gardell*, et al.*, 2007]. We went on to examine the effect of PAI–749 on the lysis of whole blood model thrombi formed under flow conditions, as well as the effect on model thrombi generated from purified plasma proteins. These systems are well validated and have previously been shown to be sensitive to the effects of PAI–1 [Mutch*, et al.*, 2007]. Indeed, a neutralising antibody directed against PAI–1, served as a positive control in each set of experiments and was shown to accelerate

lysis. However, consistent with the *ex vivo* studies, the addition of PAI–749 failed to accelerate lysis under any of the conditions used, despite its addition at concentrations well in excess of those found to produce an antithrombotic effect in pre-clinical animal models.

We employed the Badimon Chamber as an established and validated *ex vivo* clinical model of arterial injury and thrombosis that can be applied to the clinic. It has principally been used to evaluate the effects of novel antithrombotic regimens [Lev*, et al.*, 2004; Lev*, et al.*, 2006; Sarich*, et al.*, 2003; Zafar*, et al.*, 2007] and has a number of advantages over other techniques suitable for use in man. These include assessing thrombus in whole flowing blood, under rheological conditions similar to those of a coronary artery, and using a biological substrate mimicking deep arterial wall injury. Using this model, we administered exogenous t–PA in a reliable, predictable and reproducible manner allowing the demonstration of a dose-dependent reduction in thrombus area. This is consistent with the dose-dependent increase in plasma t–PA and D-dimer concentrations that correlated with the change in thrombus area.

Whilst there are a number of advantages to this model, there are also potential limitations. One issue is the time available for interaction of the test compound with flowing blood before arrival at the thrombogenic surface in the Badimon chamber. Although PAI–749 is a small molecule (MW 502 Da) that rapidly inhibits PAI–1 *in vitro* [Gardell*, et al.*, 2007], the time for interaction between PAI–749 and PAI–1 in our system is short (~2 s) and it is possible that despite the high plasma concentration of PAI–749 achieved, interaction time is not sufficient to allow effective inhibition of PAI–1. However, given that PAI–749 had no effect in any of the *in vitro* assays

performed, all of which involved significant incubation periods, the interaction time alone is unlikely to explain the lack of efficacy. A second potential limitation is that due to the quantity of antibody that was needed, it was not feasible to include a neutralising antibody directed against PAI–1 to serve as a positive control in the *ex vivo* studies.

Although previous *in vivo* studies of PAI–749 have employed well-established techniques [J. Hennan, 2006], there are a number of potentially important differences from the present study. The prior animal models used chemical and electrolytic injury to induce thrombus formation in the presence of an intact intima. In one of the models, PAI–749 was administered orally 90 min prior to vessel injury with assessments of blood flow and thrombus weight continuing for at least 40 min [J. Hennan, 2006]. In another study [J. Hennan, 2006], the compound was administered and thrombus assessed 24 h later. In the current clinical study, PAI–749 was added directly to blood with thrombus formation being assessed over only 5 min. Moreover, the principal aim in this study was to investigate the ability of PAI–749 to reduce thrombus formation in the presence and absence of exogenous t–PA; conditions that have not been previously explored.

We achieved the desired plasma PAI–749 concentrations in the Badimon chamber: 50-fold greater than that required to produce efficacy in pre-clinical studies. Thus it is unlikely that inadequate dosing is an explanation for our findings. Why then did we not see an effect? Most of the PAI–1 present in plasma complexes with, and is stablised by, vitronectin [Declerck*, et al.*, 1988; Wiman*, et al.*, 1988]. In previous *in vitro* studies, vitronectin appeared to prevent PAI–749 from binding to PAI–1 and

thereby blocked its inhibitory activity [Gardell*, et al.*, 2007]. However, an antithrombotic effect of PAI–749 was observed in the preclinical models and this may reflect the capacity of orally administered PAI–749 to interact and inhibit newly secreted free PAI–1 prior to it complexing with vitronectin. In the current study, where PAI–749 was administered directly into the blood of the extracorporeal circuit of the Badimon Chamber, there may not be an opportunity for the compound to interact with unbound, free PAI–1.

Hennan *et al.* studied the effect of another small molecule PAI–1 inhibitor, PAI–039, using a rat model of thrombosis [Hennan*, et al.*, 2008]. They noted an increase in plasma PAI–1 antigen in both treated and control animals following ferric chloride induced arterial injury. In the control animals, there was a corresponding increase in PAI–1 activity that was suppressed in the treated animals. Hennan *et al.* suggest that during vascular injury *in vivo*, a pool of unbound PAI–1 is released that is indeed vulnerable to inhibition. Clearly, the *in vitro* systems we employed here do not involve vessel injury. As discussed, although the thrombogenic substrate within the Badimon Chamber consists of porcine tunica media, the vessel is surgically prepared in advance of the studies before being subsequently frozen prior to use. Thus, such injury may not recreate the conditions necessary to generate unbound PAI–1. Finally, given that the source of the PAI–1 generated during vessel injury is not clear, and may derive from the vessel wall itself, the porcine origin of the vessels may also be relevant.

Other studies using PAI–039 have also suggested the importance of vitronectin and that this may prevent PAI–749 binding to PAI–1 [Gorlatova*, et al.*, 2007; Leik*, et al.*,

2006]. In an attempt to investigate the potential role of vitronectin, we examined the effect of PAI–749 on the lysis of fibrin clots generated from purified human plasma proteins. This allowed the effect of PAI–749 to be assessed in the presence and absence of vitronectin. This approach also failed to demonstrate an effect of PAI–749 on t–PA mediated lysis, suggesting that the presence of vitronectin alone under these conditions is not sufficient to explain the lack of compound efficacy.

Under a single set of experimental conditions in one of the *in vitro* studies, we observed a small but statistically significant *decrease* in the rate of fibrinolysis in the presence of PAI–749 (Figure 1A). Although we cannot rule out the possibility that PAI–749 has a negative effect on fibrinolysis through an as yet unrecognised mechanism, given that we employed a variety of different models and failed to detect such an effect under any other conditions, we believe this is highly unlikely and is more likely to represent a type I error.

One further explanation for the observed lack of efficacy may relate to species differences such that whilst effective in *in vitro* systems using purified human components, PAI–749 is less effective when assessed in more complex human plasma and whole blood based systems. This situation has arisen previously for a number of compounds with antithrombotic efficacy in preclinical models that subsequently failed to show efficacy in man [Cox*, et al.*, 1992; Hara*, et al.*, 1995]. However, consistent with previous work [Biemond*, et al.*, 1995; Levi*, et al.*, 1992], the ability of a monoclonal antibody against PAI–1 to accelerate fibrinolysis *in vitro* suggests that whilst PAI–749 lacks efficacy in human blood based systems, the inhibition of PAI–1 remains a potential therapeutic target worthy of further exploration.

5.5.1 CONCLUSIONS

Taken together, our data, generated in a range of complementary human plasma and whole blood-based *in vitro* and *ex vivo* systems, suggest that PAI–749 has no measurable effect on t–PA mediated fibrinolysis. These findings are in contrast to those previously published in animal models where PAI–749 was able to limit thrombus formation and enhance fibrinolysis *in vivo*. This disparity is likely to be due to either a failure of PAI–749 to inhibit human PAI–1 to an extent that yields a measurable response in the current systems, or a failure of selective inhibition of PAI–1 to result in an appreciable enhancement of fibrinolysis in man. The inability to monitor PAI–749 pharmacodynamically and the lack of an antithrombotic effect in these various human model systems suggest that PAI–749 may not be a useful therapeutic agent for the prevention and treatment of atherothrombotic disease in man. However, it is also possible that these systems do not accurately reflect the ability of small molecules to inhibit PAI–1 *in vivo*.

CHAPTER 6

DIESEL EXHAUST INHALATION INCREASES THROMBUS FORMATION IN MAN

Published by **Lucking AJ**, Lundback M, Mills NL, Faratian D, Barath SL, Pourazar J, Cassee FR, Donaldson K, Boon NA, Badimon JJ, Sandstrom T, Blomberg A, Newby DE. *Eur Heart J*. 2008 Dec; 29(24):3043-51

6.1 SUMMARY

Although the mechanism is unclear, exposure to traffic-derived air pollution is a trigger for acute myocardial infarction. The aim of this study was to investigate the effect of diesel exhaust inhalation on platelet activation and thrombus formation in man. In a double-blind randomised crossover study, 20 healthy volunteers were exposed to dilute diesel exhaust (350 $\mu g/m^3$) and filtered air. Thrombus formation, coagulation, platelet activation and inflammatory markers were measured at two and six hours following exposure. Thrombus formation was measured using the Badimon *ex vivo* perfusion chamber. Platelet activation was assessed by flow cytometry. Compared to filtered air, diesel exhaust inhalation increased thrombus formation under low and high shear conditions by 24% (change in thrombus area 2229 μm^2, 95% CI 1143 to 3315 μm^2, P=0.0002) and 19% (change in thrombus area 2451 μm^2, 95% CI 1190 to 3712 μm^2, P=0.0005) respectively. This increased thrombogenicity was seen at two and six hours, and using two different diesel engines and fuels. Diesel exhaust also increased platelet-neutrophil and platelet-monocyte aggregates by 52% (absolute change 6%, 95% CI 2 to 10%, P=0.01) and 30% (absolute change 3%, 95% CI 0.2 to 7%, P=0.03) respectively at two hours following exposure compared to filtered air.

Inhalation of diesel exhaust increases *ex vivo* thrombus formation and causes *in vivo* platelet activation in man. These findings provide a potential mechanism linking exposure to combustion-derived air pollution with the triggering of acute myocardial infarction.

6.2 INTRODUCTION

Chronic exposure to air pollution is a major cause of cardiovascular morbidity and mortality worldwide [Brunekreef, *et al.*, 2002]. Recently, exposure to traffic-derived air pollution has been associated with the triggering of acute MI [Peters, *et al.*, 2001; Peters, *et al.*, 2004]. Whilst air pollution consists of a heterogeneous mixture of gaseous and particulate matter, adverse cardiovascular events are most strongly associated with exposure to fine particulate matter (diameter <2.5 μm, $PM_{2.5}$) [Dockery, *et al.*, 1993; Miller, *et al.*, 2007]. An important component of $PM_{2.5}$ is nanoparticulate matter generated during the combustion of diesel fuel [Charron, *et al.*, 2005]. These particles, with an aerodynamic diameter ≤100 nm, readily deposit within human alveoli and possess a considerable surface area that may contribute to their biological toxicity [Donaldson, *et al.*, 2002].

Despite the strength and consistency of observational data, the pathophysiological mechanisms linking air pollution with adverse cardiovascular events remain unclear. They have been proposed to include endothelial dysfunction [Mills, *et al.*, 2007; Mills, *et al.*, 2005; Tornqvist, *et al.*, 2007], myocardial ischemia [Mills, *et al.*, 2007], altered autonomic function [Gold, *et al.*, 2000], systemic inflammation [Tornqvist, *et al.*, 2007] and platelet activation [Nemmar, *et al.*, 2003]. Thrombosis plays a central role in the pathogenesis of atherosclerosis. As well as contributing to atherogenesis, thrombosis at the site of a disrupted coronary arterial plaque may cause acute vessel occlusion resulting in an acute coronary syndrome (ACS). In pre-clinical studies employing a thrombotic vascular injury model, tracheal instillation of diesel exhaust particles caused platelet activation and increased arterial and venous thrombus formation [Nemmar, *et al.*, 2003]. Given that exposure to combustion-derived pollutants appears to act as a trigger for MI, and that the majority of such events are

due to thrombus formation at the site of an atheromatous plaque, we hypothesised that exposure to diesel exhaust would increase *in vivo* platelet activation and enhance thrombus formation in an *ex vivo* clinical model of arterial injury.

6.3 METHODS

6.3.1 SUBJECTS

Twenty healthy non-smokers aged between 21 and 44 years old were enrolled into the study (Table 6.1). The study was performed with the approval of local research ethics committees, in accordance with the Declaration of Helsinki and the written informed consent of all volunteers. Volunteers were recruited using advertisements and from local healthy volunteer databases. Exclusion criteria were the use of regular medication or clinical evidence of atherosclerosis, arrhythmias, diabetes mellitus, hypertension, renal or hepatic impairment, asthma, occupational exposure to air pollution, intercurrent infective disease or any other clinically significant illness. Subjects had normal lung function and reported no symptoms of respiratory tract infection within the six-week period preceding the study.

	Value
Age (years)	26±5
Height (m)	1.8±0.1
Weight (kg)	73±3
Body Mass Index (kg/m^2)	22±1
Pulse (bpm)	58±2
Systolic Blood Pressure (mmHg)	120±3
Diastolic Blood Pressure (mmHg)	71±2
Forced Expiratory Volume in One Second (FEV1, L)	4.9±0.1
% predicted FEV1	108±3
Forced Vital Capacity (FVC, L)	5.8±0.2
% predicted FVC	103±3

Data are presented as mean±standard deviation.

Pooled data from protocols 1 and 2 (n=20)

Table 6.1 *Baseline Volunteer Characteristics*

6.3.2 STUDY DESIGN

Subjects attended on two occasions at least one week apart and received either filtered air or dilute diesel exhaust in a double-blind randomised crossover design. Exposures were performed at separate dedicated exposure facilities by technical staff with no involvement in the clinical studies. The order of the exposures was randomised based on an independently determined exposure protocol. Subjects remained indoors following exposures to minimise confounding effects of ambient air pollution. The primary endpoint was the *ex vivo* thrombus formation. Secondary endpoints were *in vivo* platelet activation assessed by flow cytometry and changes in haematological and coagulation variables and soluble markers of inflammation.

Clinical studies were performed in dedicated clinical research facilities by clinical staff blinded to exposure allocation. Based on previous vascular and inflammatory studies [Mills*, et al.*, 2005], initial thrombosis studies were performed six hours after exposure in eight subjects (Protocol 1). In light of the findings, further thrombosis and flow cytometric studies were performed at two and six hours in a separate cohort of 12 subjects (Protocol 2). The second protocol was designed to confirm the initial findings, assess temporal effects, investigate potential mechanisms and determine if the initial findings were reproducible with a different type of diesel exposure.

Protocol 1

Exposures were performed for two hours in a mobile ambient particle concentrator exposure laboratory (MAPCEL) in Edinburgh UK. During exposures, subjects performed moderate exercise (minute ventilation 25 L/min/m^2) on a bicycle ergometer for 15 minutes alternated with 15-minute rest periods. Temperature and humidity in

the chamber were controlled at 22ºC and 50% respectively. Diesel exhaust was generated by an idling engine (type F3M2011, 2.2 L, 500 rpm; Deutz, Germany) using gas oil (Petroplus Refining, UK). Over 90% of the exhaust fumes were shunted away, with the remainder being diluted with air and fed into the exposure chamber at a steady-state concentration. Air in the chamber was continuously monitored with exposures standardised using continuous measurement of nitrogen oxide (NO_x) concentrations to deliver a particulate concentration of 350 $\mu g/m^3$. There was little variation in particle mass (348±68 $\mu g/m^3$), particle number (1.2±0.1 x $10^6/cm^3$), NO_x (0.58±0.03ppm), NO_2 (0.23±0.02ppm), NO (0.36±0.02ppm), CO (3.54±0.76ppm) and total hydrocarbon (2.8±0.1 $\mu g/m^3$) concentrations between exposures.

Protocol 2

In Umeå Sweden, subjects were exposed for one hour in a purpose-built diesel exposure chamber according to a standard protocol, as described previously [Mills*, et al.*, 2005; Rudell*, et al.*, 1994]. Diesel exhaust was generated by an idling Volvo engine (TD45, 4.5 L, 680 rpm) using Gasoil E10 (Preem, Sweden) as described previously [Mills*, et al.*, 2005; Rudell*, et al.*, 1994]. During exposures, subjects performed periods of exercise as described above. Exposures were standardised using continuous measurement of NO_x to deliver a particulate concentration of 350 $\mu g/m^3$. There was little variation in particle mass (330±12 $\mu g/m^3$), particle number (1.26±0.01 x $10^6/cm^3$), NO_x (2.78±0.03ppm), NO_2 (0.62±0.01ppm), NO (2.15±0.03ppm), CO (3.08±0.12ppm) and total hydrocarbon (1.58±0.16 $\mu g/m^3$) concentrations between exposures.

6.3.3 *EX VIVO* THROMBOSIS STUDIES

Thrombus formation was measured using the Badimon Chamber as described in section 2.3.

Immediately after each study, porcine strips with thrombus attached were removed and fixed in 4% paraformaldehyde. Strips were wax embedded, sectioned and stained with Masson's Trichrome. Images were acquired at ×20 magnification and thrombus area measured using an Ariol image acquisition system (Applied Imaging, USA) and Image-Pro Plus software (Media Cybernetics, USA) by a blinded operator. Results from at least six sections were averaged to determine thrombus area for each chamber as described previously [Badimon*, et al.*, 1987; Osende*, et al.*, 2001; Wahlander*, et al.*, 2006].

6.3.4 FLOW CYTOMETRY

Samples were obtained at two and six hours, immediately prior to each thrombosis study, and processed according to previously described protocols [Harding*, et al.*, 2006]. In brief, blood was taken from an antecubital vein using a 21-gauge cannula and anticoagulated with D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (PPACK, 75 μm; Cambridge Biosciences, UK). Samples were not analysed unless venesection achieved rapid and uninterrupted blood flow. Five minutes after sample collection, samples were stained with the following conjugated monoclonal antibodies: PE-conjugated CD14 (Dako, Denmark), PE-conjugated CD62P, PE-conjugated CD154 (Becton-Dickinson, UK); PE-conjugated CD11b, PE-conjugated CD40, FITC-conjugated CD42a, FITC-conjugated CD14 (Serotec, USA); and appropriate control isotypes. All antibodies were diluted 1:20. Once stained, samples

were incubated for 20 minutes at room temperature to identify P-selectin and CD40L on the platelet surface and CD40 on the monocyte surface. Monocyte and platelet-leucocyte samples were fixed with FACS-Lyse (Becton-Dickinson, UK). Platelet samples were fixed with 1% paraformaldehyde. Samples were analysed within 24 hours using a FACScan flow cytometer (Becton-Dickinson, UK). Platelet–monocyte and platelet–neutrophil aggregates were defined as monocytes or neutrophils positive for CD42a. Data analysis was performed using FlowJo (Treestar, USA).

6.3.5 BLOOD SAMPLING

Samples were obtained before exposure and at two and six hours. Samples were analysed for total white cell count, differential cell count and platelets by an autoanalyser. Plasma interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), soluble CD40 ligand (sCD40L), soluble P-selectin, intercellular adhesion molecule-1 (ICAM-1) and C-reactive protein (CRP) were measured with commercially available ELISAs (R&D Systems, UK). Prothrombin time (PT, reagents from Medirox, Sweden) and activated partial thromboplastin time (aPTT, reagents from Dade Behring, USA) were measured using a CA-7000 analyser (Sysmex, Japan).

6.3.6 STATISTICAL ANALYSIS

Data presented are pooled from protocols 1 and 2 unless otherwise stated. Continuous variables are reported as mean±standard deviation (SD). Statistical analysis was performed in Excel (Microsoft Corporation, USA) using a modified *t*-test (two-sided) to account for potential period effects [Pocock, 1983]. Statistical significance was taken at $P<0.05$.

6.4 RESULTS

Exposures and clinical studies were well-tolerated with no adverse symptoms reported. All volunteers completed both study visits.

Total leucocyte, monocyte and platelet counts, PT and aPTT were unaltered following dilute diesel exhaust and filtered air (Table 6.2). Whilst neutrophil count appeared to increase and lymphocyte count appeared to decrease following both exposures, there were no differences in the magnitude of these changes following dilute diesel exhaust compared with filtered air (Table 6.2).

						P values	
	Before Exposure	***2 Hours***	***6 Hours***	***Δ 2 Hours***	***Δ 6 Hours***	***2 Hours***	***6 Hours***
FILTERED AIR							
Leucocytes ($\times10^9$ cells/L)	5.44±1.42	5.27±1.16	5.75±1.07	-0.18±1.08	0.26±1.19	….	….
Lymphocytes ($\times10^9$ cells/L)	2.17±0.67	1.92±0.52	1.68±0.43	-0.48±0.46	-0.51±0.53	….	….
Neutrophils ($\times10^9$ cells/L)	2.62±0.69	3.00±0.75	3.51±0.91	0.42±0.68	0.89±0.86	….	….
Monocytes ($\times10^9$ cells/L)	0.47±0.16	0.42±0.14	0.41±0.16	-0.06±0.11	-0.06±0.10	….	….
Platelets ($\times10^9$ cells/L)	223±38	221±35	223±30	-1.80±18	1.79±22	….	….
INR‡	1.00±0.06	1.03±0.07	1.04±0.07	0.02±0.04	0.03±0.05	….	….
aPTT‡ (sec)	28.9±0.91	29.1±0.89	28.7±0.90	0.28±0.47	-0.20±0.58	….	….
DIESEL EXHAUST							
Leucocytes ($\times10^9$ cells/L)	5.45±1.22	5.51±1.54	5.53±1.31	0.06±1.34	0.07±1.41	0.54	0.52
Lymphocytes ($\times10^9$ cells/L)	2.20±0.73	1.67±0.55	1.61±0.42	-0.53±0.36	-0.59±0.58	0.60	0.30
Neutrophils ($\times10^9$ cells/L)	2.60±0.64	3.26±1.42	3.38±1.17	0.68±1.27	0.81±1.17	0.90	0.90
Monocytes ($\times10^9$ cells/L)	0.49±0.11	0.45±0.12	0.45±0.13	-0.07±0.09	-0.08±0.08	0.69	0.60
Platelets ($\times10^9$ cells/L)	227±38	223±47	218±31	-4.15±21	-3.74±16	0.72	0.32
INR‡	1.02±0.06	1.02±0.08	1.03±0.08	0.01±0.03	0.01±0.03	0.55	0.26
aPTT‡ (sec)	29.3±1.39	29.22±0.87	29.2±0.89	-0.07±0.86	-0.06±0.74	0.22	0.83

Data are presented as mean±standard deviation.

Pooled data from protocols 1 and 2, (n=20, except ‡ unavailable for protocol 1).

P values are for the comparison of diesel exhaust *versus* filtered air

aPTT - activated partial thromboplastin time, INR - international normalised ratio of prothrombin time

Table 6.2 *Effects of Dilute Diesel Exhaust on Heamatological and Coagulation Variables*

6.4.1 MARKERS OF INFLAMMATION AND PLATELET ACTIVATION

There was a heterogenous cytokine response following both dilute diesel exhaust and filtered air exposures (Table 6.3, data from protocol 2). Changes in plasma TNF-α, IL-6, CRP and soluble ICAM-1 concentrations were similar following both exposures. Following dilute diesel exhaust exposure, plasma sCD40L concentrations were increased at two hours (P=0.003 *versus* filtered air) and the fall at six hours following filtered air exposure was attenuated (P=0.011 *versus* filtered air). Similarly, the fall in plasma soluble P-selectin concentration at six hours following filtered air exposure was attenuated following diesel exhaust exposure (P=0.003).

						P values	
	Before Exposure	***2 Hours***	***6 Hours***	***Δ 2 Hours***	***Δ 6 Hours***	***Δ 2 Hours***	***Δ 6 Hours***
FILTERED AIR							
TNF-α (pg/mL)	0.62±0.73	….	0.36±0.32	….	-0.26±0.30	….	….
IL-6 (pg/mL)	0.60±0.76	….	0.88±0.80	….	0.28±1.1	….	….
CRP (mg/L)	1.02±0.66	1.09±1.42	1.13±1.64	0.07±0.17	0.12±0.16	….	….
Soluble CD40L (pg/mL)	66±29	75±8	41±14	9.00±29	-20±26	….	….
Soluble P-selectin (mg/mL)	61±27	37±8	44±18	-25±22	-18±26	….	….
Soluble ICAM-1 (mg/mL)	278±65	170±25	271±69	-109±56	-6.40±66	….	….
DIESEL EXHAUST							
TNF-α (pg/mL)	0.55±0.41	….	0.57±0.44	….	0.06±0.44	….	0.11
IL-6 (pg/mL)	0.27±0.19	….	0.78±0.61	….	0.52±0.66	….	0.51
CRP (mg/L)	0.67±0.65	0.66±0.94	0.65±0.85	-0.01±0.10	-0.02±0.04	0.48	0.30
Soluble CD40L (pg/mL)	48±16	78±8	42±10	30±15	-0.85±18	0.01	0.01
Soluble P-selectin (mg/mL)	51±18	37±10	54±14	-14±13	2.89±16	0.11	0.003
Soluble ICAM-1 (mg/mL)	263±56	181±33	280±58	-82±37	17±59	0.49	0.64

Data are presented as mean±standard deviation.

Data from protocol 2 (n=12)

P values are for the comparison of diesel exhaust *versus* filtered air

TNF-α – tumour necrosis factor-α, IL-6 – interleukin-6, CRP – C reactive protein, CD40L – CD40 ligand, ICAM-1 – intercellular adhesion molecule-1

Table 6.3 *Effects of Dilute Diesel Exposure on Markers of Inflammation and Platelet Activation*

6.4.2 FLOW CYTOMETRY

Monocyte surface expression of CD40, and platelet surface expression of CD40L and P-selectin, were similar following dilute diesel exhaust and filtered air exposure (data not shown). Compared to filtered air, diesel exhaust exposure increased platelet-neutrophil and platelet-monocyte aggregates at two hours by 52% (absolute change 6%, 95% CI 2 to 10%, P=0.01) and 30% (absolute change 3%, 95% CI 0.2 to 7%, P=0.03) respectively (Figure 6.1, data from protocol 2). There was a trend towards similar increases at six hours although these were not statistically significant.

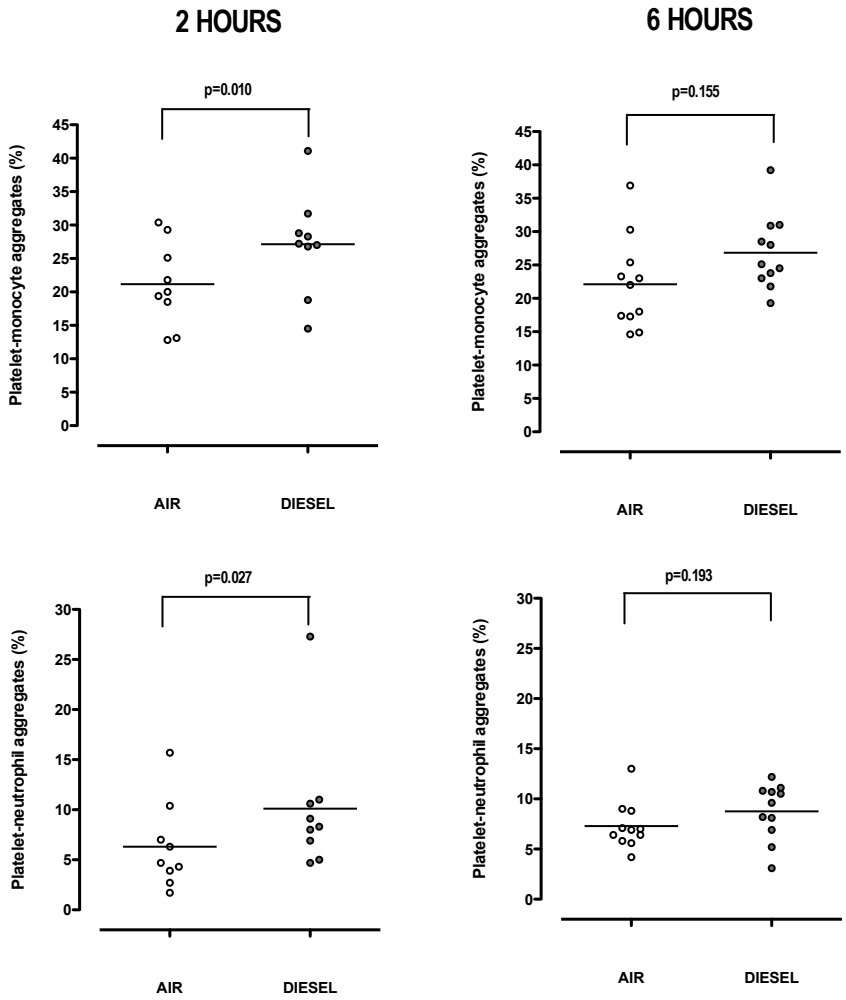

Figure 6.1 *Platelet-leucocyte aggregates two and six hours following dilute diesel exhaust (●) and filtered air (○) exposures (Protocol 2).*

6.4.3 THROMBUS FORMATION

Thrombus formation increased following dilute diesel exhaust by 23% in the low-shear (change in thrombus area 1941 μm^2, 95% CI 873 to 3008 μm^2, P=0.002) and 21% in the high-shear chamber (change in thrombus area 2916 μm^2, 95% CI 1365 to 4466 μm^2, P=0.001) compared to filtered air at six hours (Figure 6.2).

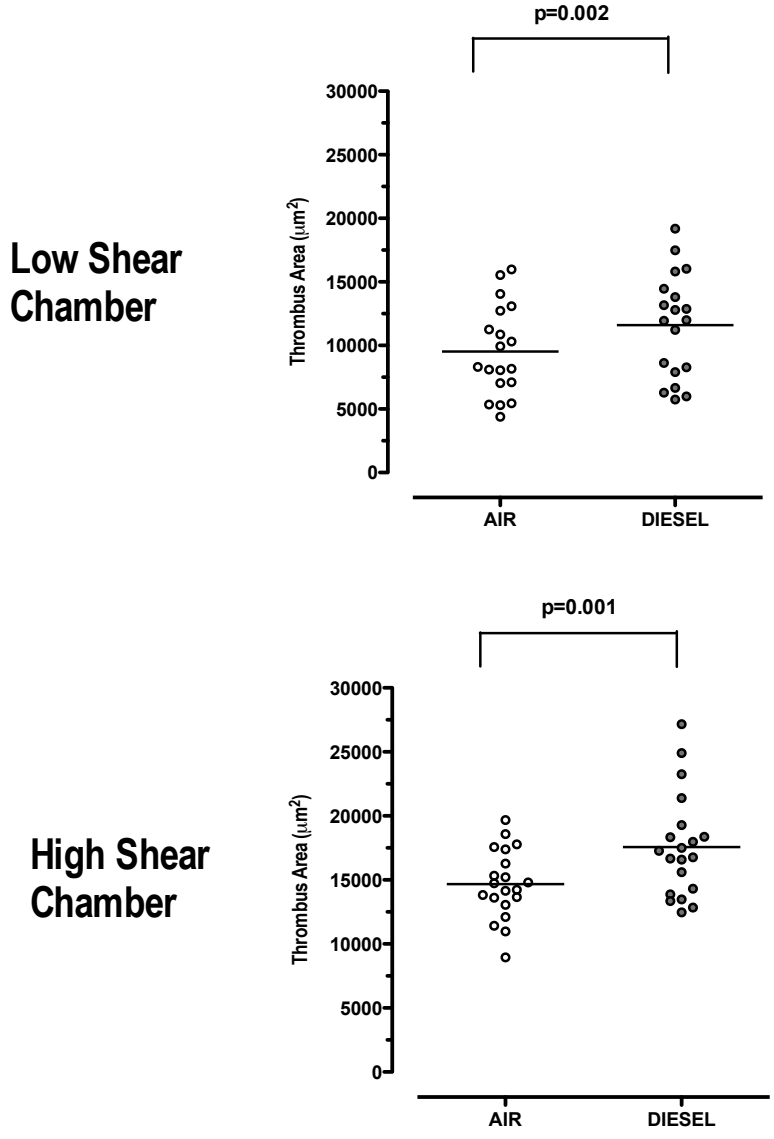

Figure 6.2 *Thrombus formation six hours following dilute diesel exhaust (●) and filtered air (○) exposures (Protocols 1 and 2).*

In Protocol 2, thrombus formation at two hours increased by 27% in the low- and 21% in the high-shear chamber following dilute diesel compared to air (change in thrombus area 2772 μm^2, 95% CI 879 to 4721 μm^2, P=0.04 and change in thrombus area 2312 μm^2, 95% CI 597 to 4026 μm^2, P=0.014 respectively; Figure 6.3). Likewise, thrombus formation at six hours was increased by 22% (change in thrombus area 2254 μm^2, 95% CI 244 to 3924 μm^2, P=0.033) in the low-shear chamber and appeared to increase (13%; change in thrombus area 1467 μm^2, 95% CI -230 to 3163 μm^2, P=0.083) in the high-shear chamber (Figure 6.3, data from protocol 2).

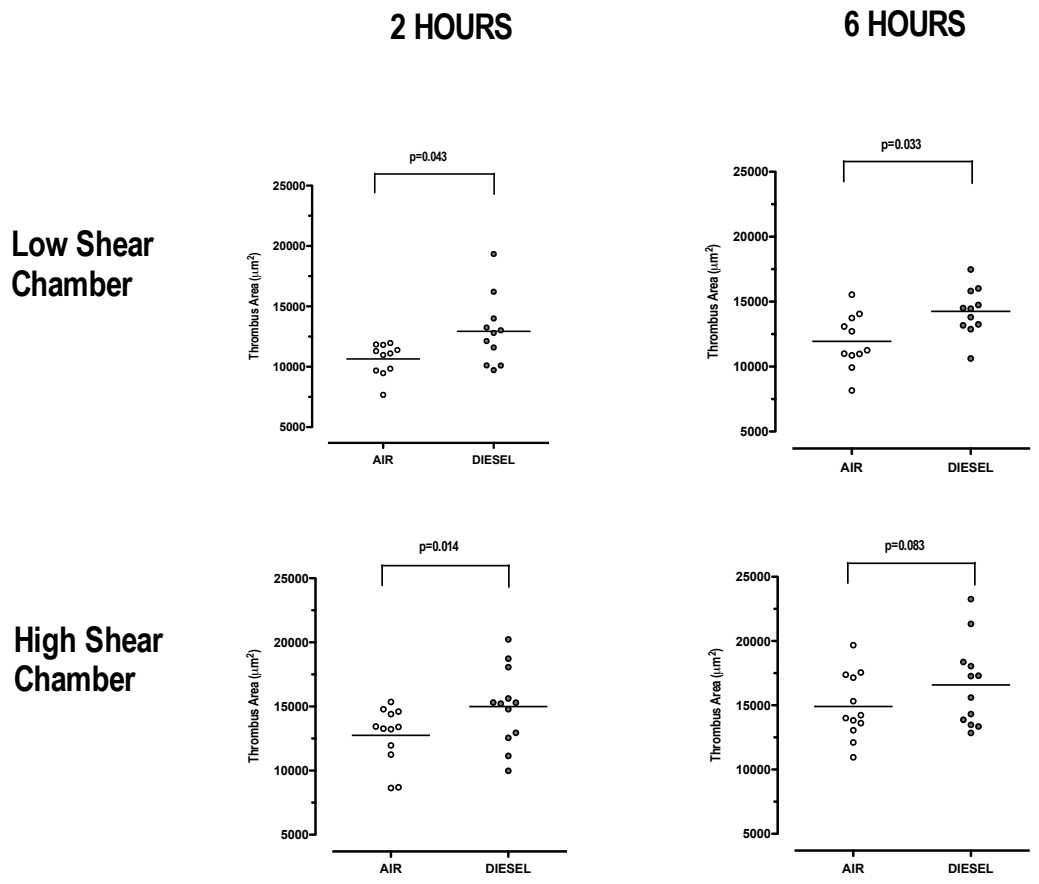

Figure 6.3 *Thrombus formation two and six hours following dilute diesel exhaust (●) and filtered air (○) exposures (Protocol 2).*

6.5 DISCUSSION

Short-term exposure to traffic-derived air pollution is associated with acute cardiovascular events [Peters*, et al.*, 2001; Peters*, et al.*, 2004]. This is the first study to demonstrate that inhalation of diesel exhaust, a common urban air pollutant, causes platelet activation and enhances thrombus formation in man. This provides a plausible mechanism linking exposure to particulate air pollution with acute cardiovascular events including MI.

6.5.1 EFFECT OF DIESEL EXHAUST ON THROMBOSIS

Despite the suggestion from observational studies that exposure to combustion-derived air pollution is associated with MI, few studies have examined whether controlled exposure alters thrombotic potential. Developing a reproducible *in vivo* model of thrombosis for use in human studies is challenging. We therefore used the Badimon chamber as a validated *ex vivo* model of arterial injury and thrombosis [Badimon*, et al.*, 1987]. It has previously been used to evaluate the effects of novel antithrombotic regimens and has a number of advantages over other techniques [Badimon*, et al.*, 1987; Osende*, et al.*, 2001; Wahlander*, et al.*, 2006]. It allows the measurement of thrombus formation in native (non-anticoagulated) whole blood triggered by exposure to a physiologically relevant substrate, and under flow conditions mimicking those in diseased coronary arteries. Thus, this is a particularly relevant model as it broadly simulates the intra-arterial conditions following spontaneous or iatrogenic plaque disruption within the coronary vasculature.

Taken together with our previous finding that dilute diesel exhaust exposure impairs endothelial t-PA release [Mills*, et al.*, 2005], we suggest that enhanced thrombus

formation is an important mechanism that may explain the association of MI shortly after traffic pollution exposure.

6.5.2 EFFECT OF DIESEL EXHAUST ON PLATELET ACTIVATION

Platelets are key components of arterial thrombosis. Shortening of closure times in a platelet function analyser have been observed following tracheal instillation of diesel exhaust particles in hamsters [Nemmar*, et al.*, 2003] and their addition to human blood enhances platelet aggregation [Radomski*, et al.*, 2005]. Here we used flow cytometry to measure surface expression of platelet and leucocyte activation markers as well as platelet-leucocyte aggregates, a technique increasingly recognised as the gold standard measure of *in vivo* platelet activation, including in patients with ACS [Freedman*, et al.*, 2002].

We observed an increase in platelet-neutrophil and platelet-monocyte aggregates after dilute diesel exhaust exposure suggesting that enhanced thrombus formation was mediated through platelet activation. These findings are consistent with an increase in circulating platelet-leucocyte aggregates observed in women exposed to biomass smoke [Ray*, et al.*, 2006]. In addition, tracheal instillation of carbon nanotubes increased platelet-leucocyte aggregates and thrombus formation in a murine model of vascular injury [Nemmar*, et al.*, 2007]. Interestingly, blockade of P-selectin abrogated platelet-leucocyte aggregation and thrombus formation, suggesting that P-selectin serves as a link between pulmonary inflammation, systemic inflammation and enhanced thrombogenicity. Although platelet-monocyte binding is principally dependent on P-selectin, we did not observe an increase in platelet surface expression of P-selectin. However, in patients with MI, platelet-monocyte aggregates have been

shown to be a more sensitive marker of platelet activation than P-selectin [Michelson, *et al.*, 2001] as P-selectin is rapidly shed from the platelet surface [Michelson, *et al.*, 1996]. Exposure to dilute diesel exhaust also increased plasma sCD40L levels. This is in keeping with studies that demonstrated upregulation of the CD40/CD40L pathway in cigarette smokers [Harding, *et al.*, 2004] and following exposure to ultrafine particles [Becker, *et al.*, 2003; Ruckerl, *et al.*, 2007]. As platelets contain large amounts of CD40L that is released following activation [Phipps, *et al.*, 2001], the increase in sCD40L we observed further strengthens the argument that the enhanced thrombus formation observed was driven principally by platelet activation.

It is not possible from our study to determine the mechanism of platelet activation. Debate remains as to whether inhaled components of diesel exhaust can translocate into the systemic circulation [Mills, *et al.*, 2006; Nemmar, *et al.*, 2002] to mediate direct effects on blood and vascular components. A substantial body of evidence supports a role for oxidative stress and inflammation in mediating the adverse effects of air pollution [Donaldson, *et al.*, 2005]. Although we did not observe an increase in cellular or soluble inflammatory markers, this does not preclude a role for factors not assessed here. The ability of diesel exhaust exposure to cause pulmonary inflammation is not in doubt [Salvi, *et al.*, 1999] and we have demonstrated previously that diesel particles are capable of generating free radicals [Tornqvist, *et al.*, 2007] which may activate platelets by reducing endothelial and platelet derived nitric oxide and antioxidants.

6.5.3 EFFECT OF DIESEL EXHAUST ON COAGULATION

A number of previous ambient and controlled exposure studies have evaluated the association between plasma concentrations of coagulation factors and particulate air pollution with mixed results. Whilst some have demonstrated increased levels of fibrinogen [Ghio*, et al.*, 2000; Mutlu*, et al.*, 2007; Pekkanen*, et al.*, 2000] and von Willebrand factor [Ruckerl*, et al.*, 2006], other studies measuring the same factors have failed to show any association with particulate exposure [Elder*, et al.*, 2004; Gong*, et al.*, 2003; Ruckerl*, et al.*, 2006]. This apparent disparity may well be explained by variations in study design and perhaps more importantly, the type of exposure investigated. Two previous studies have investigated the effect of controlled diesel exhaust exposure on coagulation factors in man with neither demonstrating a significant effect [Blomberg*, et al.*, 2005; Carlsten*, et al.*, 2007]. Although a recent observational study reported a small reduction in PT associated with ambient exposure to PM_{10} [Baccarelli*, et al.*, 2007], we found no effect on PT or aPTT following exposure to diesel exhaust. Despite higher effective particulate matter concentrations, our findings are in keeping with previous controlled diesel exposure studies that failed to demonstrate changes in fibrinogen, von Willebrand factor, D-dimer, pro-thrombin fragment 1 and 2, tissue-plasminogen activator and plasminogen activator inhibitor-1 [Blomberg*, et al.*, 2005; Carlsten*, et al.*, 2007].

6.5.4 POPULATION RISK AND DIESEL EXPOSURE

Diesel exhaust is an important source of combustion-derived air pollution. We have now performed a large number of inhalation exposures in healthy subjects and patients with cardiovascular disease using well-characterised systems. Particulate levels during these exposures are comparable with those in heavy traffic and

occupational settings in large cities. Here, we used two types of diesel engine and two different commercially available fuels. The particulate component was similar in both protocols. Despite differences in the method of diesel exhaust generation and the gaseous component, prothrombotic effects were consistent.

Although the overall implications of exposure to traffic-derived pollution are significant from a population perspective, individual risk is modest. Observational data support the notion that risk is greatest in those with pre-existing cardiovascular disease. Indeed, we have recently demonstrated that dilute diesel exhaust inhalation has pro-ischemic effects in patients with prior MI [Mills*, et al.*, 2007]. In the present study, we have extended these findings using a clinical model of severe arterial injury that is reflective of the intravascular conditions in a patient with a ruptured or denuded atheromatous plaque. Our findings of enhanced platelet activation and thrombus formation further highlight the potential increased propensity of 'at risk' populations to suffer adverse cardiovascular consequences following exposure to air pollution although it is not possible from this study to determine whether diesel exhaust exposure enhances thrombogenicity in patients on antiplatelet therapies.

6.5.5 CONCLUSION

Inhalation of dilute diesel exhaust causes platelet activation and increased thrombus formation in man. This study provides a plausible pathophysiological link that may explain the association between combustion-derived air pollution and acute cardiovascular events. Further work is required to clarify more precisely the mechanism of enhanced thrombogenicity and to investigate how this potentially harmful effect may be abrogated.

CHAPTER 7

PARTICLE TRAPS PREVENT ADVERSE VASCULAR AND PROTHROMBOTIC EFFECTS OF DIESEL ENGINE EXHAUST INHALATION IN MAN

Published by **Lucking AJ**, Lundbäck M, Barath SL, Mills NL, Sidhu MK, Langrish JP, Boon NA, Pourazar J, Badimon JJ, Gerlofs-Nijland ME, Cassee FR, Boman C, Donaldson K, Sandstrom T, Newby DE, Blomberg A.
Circulation. 2011 Apr 26; 123(16):1721-8

7.1 SUMMARY

In controlled human exposure studies, diesel engine exhaust inhalation impairs vascular function and enhances thrombus formation. The aim of the present study was to establish whether an exhaust particle trap could prevent these adverse cardiovascular effects in men. Nineteen healthy volunteers (mean age 25±3 years) were exposed to filtered air and diesel exhaust in the presence or absence of a particle trap for one hour in a randomised double-blind three-way crossover trial. Bilateral forearm blood flow and plasma fibrinolytic factors were assessed using venous occlusion plethysmography and blood sampling during intra-arterial infusion of acetylcholine, bradykinin, sodium nitroprusside and verapamil. *Ex vivo* thrombus formation was determined using the Badimon chamber. Compared to filtered air, diesel exhaust inhalation was associated with reduced vasodilatation and increased *ex vivo* thrombus formation under both low and high shear conditions. The particle trap markedly reduced diesel exhaust particulate number (150,000-300,000 to 30-300/cm^3; $P<0.001$) and mass (320±10 to 7.2±2.0 $\mu g/m^3$; $P<0.001$), and was associated with increased vasodilatation, reduced thrombus formation and an increase in tissue plasminogen activator release.

Exhaust particle traps are a highly efficient method of reducing particle emissions from diesel engines. Using a range of surrogate measures, the use of a particle trap prevents several adverse cardiovascular effects of exhaust inhalation in men. Given these beneficial effects on biomarkers of cardiovascular health, the widespread use of particle traps on diesel-powered vehicles may have substantial public health benefits and reduce the burden of cardiovascular disease.

7.2 INTRODUCTION

There is a robust and consistent association between air pollution and cardio-respiratory morbidity and mortality [Brunekreef*, et al.*, 2002; Dockery*, et al.*, 1993; Logan, 1953; Samet*, et al.*, 2000]. These harmful effects are most strongly associated with exposure to traffic-derived fine particles (particulate matter [PM] with a mean diameter <2.5 μm, $PM_{2.5}$) that predominantly originate from diesel engine exhaust emissions [Laden*, et al.*, 2000]. Diesel engines are popular because of their reliability, efficiency and relatively low running costs. However, they generate up to 100 times more fine particles than petroleum engines of a similar size and contribute substantially to the global burden of PM air pollution.

According to the World Health Organisation, air pollution is responsible for at least 800,000 premature deaths worldwide each year with an average one year loss of life [Cohen*, et al.*, 2005]. The long-term risk of cardiovascular death rises by 76% for each 10μg/m^3 increase in $PM_{2.5}$ [Miller*, et al.*, 2007; Peters*, et al.*, 2002]. Short-term exposure has been linked to the triggering of acute myocardial infarction [Bhaskaran*, et al.*, 2009] with patients being three times more likely to be exposed to traffic-derived air pollution in the hours before their acute myocardial infarction [Peters*, et al.*, 2001]. Despite the strength and consistency of observational studies, the underlying pathophysiological mechanisms remain unclear. Using well-characterised and controlled diesel engine exhaust exposure studies in healthy volunteers, we have previously demonstrated impaired vascular vasomotor and fibrinolytic function [Mills*, et al.*, 2005], increased arterial stiffness [Lundback*, et al.*, 2009] and enhanced *ex vivo* thrombus formation [Lucking*, et al.*, 2008]. Furthermore, in patients with coronary heart disease, we have shown that diesel engine exhaust inhalation

exacerbates exercise induced ST-segment depression during light exercise [Mills*, et al.*, 2007]. Taken together, these adverse cardiovascular effects provide important mechanisms that help to explain the detrimental health effects of air pollution exposure.

One approach to limit traffic emissions has been the introduction of diesel engine exhaust particle traps or filters. Whilst the efficiency of particle traps to reduce particle emission is >90%, particles are not completely eliminated and traps have the potential to create new and potentially more toxic particles that may outweigh the benefits of reducing the emitted particle mass [Su*, et al.*, 2008]. We therefore sought to determine whether the introduction of a particle trap would attenuate or worsen the adverse cardiovascular effects of diesel engine exhaust inhalation [Lucking*, et al.*, 2008; Lundback*, et al.*, 2009; Mills*, et al.*, 2007; Mills*, et al.*, 2005].

7.3 METHODS

Twenty-one subjects were screened and one subject was excluded at the initial screening. A further subject was excluded after randomisation due to inability to complete all exposures leaving 19 healthy non-smoking men who completed the full study protocol (see supplement Figure 7a, below [CONSORT diagram]). The study was approved by the local research ethics committee, and conducted in accordance with the Declaration of Helsinki, and with the written informed consent of all volunteers.

All subjects had normal lung function and no symptoms of upper airway infection for the four weeks prior to or during the study. Exclusion criteria were regular medication, clinical evidence of atherosclerotic vascular disease, arrhythmias, diabetes mellitus, hypertension, renal or hepatic failure, asthma, significant occupational exposure to air pollution, or intercurrent illness. All subjects abstained from caffeine containing drinks or food for at least four hours and from alcohol for 24 hours before each assessment.

7.3.1 STUDY DESIGN

The primary endpoints were endothelial vasomotor and fibrinolytic function and *ex vivo* thrombus formation. Secondary endpoints were soluble markers of inflammation and platelet activation. Exploratory endpoints were markers of arterial stiffness and airway inflammation. Sample size was determined *a priori* and based on power calculations for the primary end-points derived from our previous studies (see supplement material, below) [Lucking, *et al*., 2008; Mills, *et al*., 2005].

In a randomised double-blind three-way crossover design, subjects were exposed to filtered air, unfiltered dilute diesel engine exhaust, and dilute diesel engine exhaust that had passed through a particle trap. The order of the exposures was randomised using an independent predetermined exposure sequence. Exposures were performed at a separate dedicated exposure facility by technical staff with no involvement in the clinical studies. Clinical studies were performed in a dedicated clinical research facility by clinical staff blinded to exposure allocation. Exposures were separated by at least one week and performed in a purpose-built exposure chamber, according to a previously described standard protocol [Mills*, et al.*, 2005]. During each one-hour exposure, subjects performed moderate exercise (minute ventilation, 25 L/min/m^2 body) on a bicycle ergometer for 15 min alternated with 15 min of rest.

7.3.2 DIESEL EXHAUST

A Volvo diesel engine (Volvo TD40 GJE, 4.0 L, four cylinders) running on a Volvo standard diesel fuel (SD-VSD-10) was used to generate the diesel exhaust. The specification of the Volvo diesel fuel is similar to the European automotive standard diesel (EN590), with a sulphur content of 5-7 mg/kg and polycyclic aromatic hydrocarbon content of 2-6% by mass. The engine worked under transient speed and load conditions in accordance with the standardised European Transient Cycle that mimics real-world urban driving conditions [Barath*, et al.*]. More than 90 % of the exhaust was shunted away and the residual exhaust was mixed with filtered air (Figure 7.1).

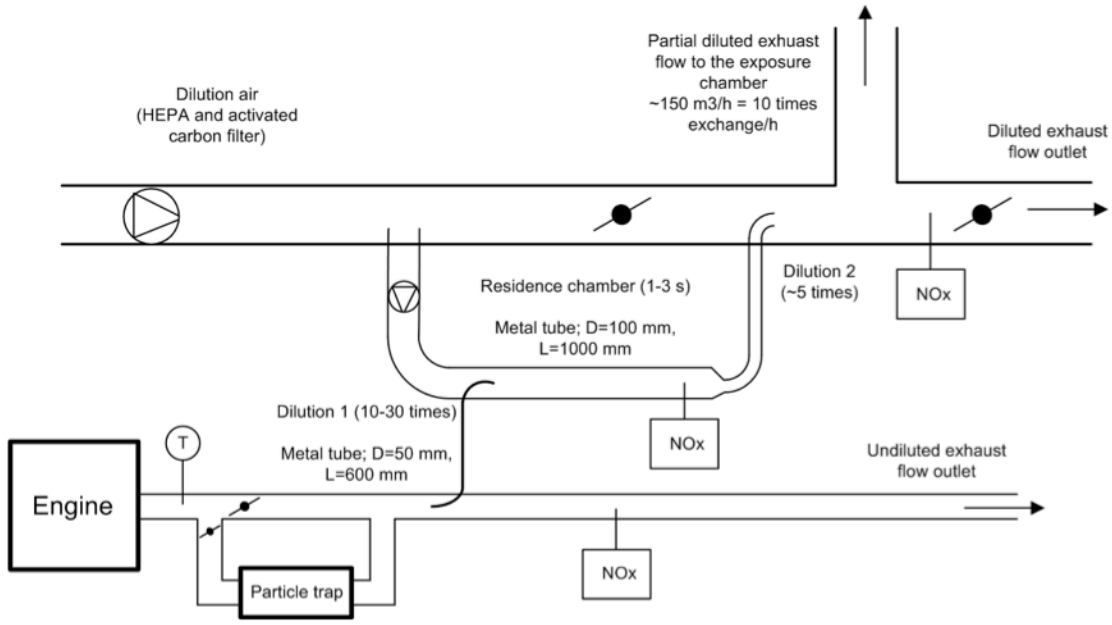

Figure 7.1 *Scheamatic illustration of the diesel exhaust set-up from the engine to the flow of diluted exhaust to the chamber.*

The concentrations of nitrogen oxides (NO, NO_2 and NO_x) in the chamber were continuously monitored together with total gaseous hydrocarbons (THC). During diesel exhaust exposures, we aimed to generate a PM mass concentration of 300 $\mu g/m^3$. This PM mass concentration was maintained for the inlet conditions of the particle trap. Actual exposure was measured gravimetrically with standard glass fibre filter sampling together with the use of a TEOM on-line instrument and in accordance with a well established protocol, as described previously [Behndig*, et al.*, 2006]. In addition, a SMPS system was used to determine fine (<1 μm) particle number concentration.

7.3.3 PARTICLE TRAP

The particle trap (DPF-CRT® [Continuously Regenerating Trap], Johnson Matthey, Royston, UK) used is an unmodified CRT filter, available commercially throughout the world as a factory-fit option or as a retrofit unit to buses and heavy goods vehicles. It is similar in design to filters produced by a number of manufacturers. It consists of a honeycomb-like complex of channels through which the exhaust is passed. A catalyst at the front of the filter oxidises part of the NO gas in the exhaust into NO_2, which flows through the particle filter and subsequently reacts with trapped carbonaceous particles to generate CO_2 and N_2. This increases NO_2 levels in the exhaust after the particle trap, without causing significant changes in total NO_x concentrations, whilst achieving an efficient reduction in particle emissions.

7.3.4 VASCULAR STUDIES

Based on data from previous exposure studies [Barath*, et al.*; Lucking*, et al.*, 2008; Mills*, et al.*, 2007; Tornqvist*, et al.*, 2007], vascular assessment was performed six to eight hours after each exposure. Assessments were performed with subjects resting supine in a quiet temperature controlled (22-24°C) room. Venous cannulae (17 gauge) were inserted into large subcutaneous veins in the antecubital fossae of both arms. The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle. After a baseline 30-min saline infusion, bradykinin at 100, 300 and 1,000 pmol/min (endothelium-dependent vasodilator that releases tissue plasminogen activator [t-PA]; Merck Biosciences, Nottingham, UK); acetylcholine at 5, 10, and 20 µg/min (endothelium-dependent vasodilator that does not release t-PA; Merck Biosciences); and sodium nitroprusside at 2, 4, and 8 µg/min (endothelium-independent vasodilator that does not release t-PA; David Bull Laboratories,

Warwick, UK) were infused for six min at each dose. The three vasodilators were given in random order, separated by a 20-min saline infusion. Verapamil was infused at 10, 30, and 100 μg/min (endothelium-independent and nitric oxide-independent vasodilator that does not release t-PA) at the end of the study protocol. The infusion of these vasodilators was undertaken to allow the assessment of distinct aspects of vascular function including nitric oxide, endothelium, fibrinolysis and vasomotion.

Forearm blood flow was measured in both infused and non-infused arms using venous occlusion plethysmography incorporating mercury-in-silicone elastomer strain gauges as described previously [Mills*, et al.*, 2005]. Heart rate and blood pressure were monitored in the non-infused arm throughout each study with a non-invasive, semi-automated oscillometric sphygmomanometer (Boso Medicus, Jungingen, Germany). Blood was drawn simultaneously from the venous cannulae in each arm at baseline and during infusion of each dose of bradykinin. Samples were collected into acidified buffered citrate (Stabilyte®, Biopool International) for t-PA assays and into citrate (BD Vacutainer) for plasminogen activator inhibitor type 1 (PAI-1) assays. Samples were kept on ice before being centrifuged at 2,000*g* for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma t-PA and PAI-1 antigen concentrations were determined by ELISA (TintElize t-PA, Biopool EIA, Trinity Biotech, Ireland; Coaliza PAI-1, Chromogenix AB, Milan, Italy). Heamatocrit was determined by capillary tube centrifugation of samples collected at baseline and during infusion of bradykinin at 1,000 pmol/min.

7.3.5 MARKERS OF INFLAMMATION

Venous blood samples were obtained before and at two, six and eight hours after exposure. Samples were analysed for total and differential cell count using an autoanalyser. Plasma interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), soluble CD40 ligand (sCD40L), soluble P-selectin, intercellular adhesion molecule-1 (ICAM-1) and C-reactive protein (CRP) were measured with commercially available enzyme-linked immunosorbant assays (R&D Systems, Abingdon, UK).

7.3.6 *EX VIVO* THROMBOSIS STUDIES

Thrombus formation was measured using the Badimon Chamber as described in section 2.3.

Immediately after each study, porcine strips with thrombus attached were removed and fixed in 4% paraformaldehyde. Strips were paraffin-wax embedded, sectioned and stained with Masson's Trichrome. Images were acquired at ×20 magnification and thrombus area measured using a semi-automated image acquisition system (Ariol, Applied Imaging, USA) by a blinded operator. Results from at least six sections were averaged to determine thrombus area for each chamber as described previously [Badimon*, et al.*, 1987; Osende*, et al.*, 2001].

7.3.7 ASSESSMENT OF ARTERIAL STIFFNESS

Please see supplement material, below

7.3.8 ASSESSMENT OF AIRWAY INFLAMMATION

Please see supplement material, below.

7.3.9 DATA ANALYSIS AND STATISTICS

Plethysmographic data were analysed as described previously [Mills*, et al.*, 2005]. Estimated net release of t-PA antigen was defined as the product of the infused forearm plasma flow (based on the mean heamatocrit and the infused forearm blood flow) and the concentration difference between the infused and non-infused arms as described previously [Mills*, et al.*, 2007; Mills*, et al.*, 2005]. Continuous variables are reported as mean ± standard error of the mean (SEM). Statistical analyses were performed with GraphPad Prism (Graph Pad Software, California, USA). All studies, data analysis and data exclusion were performed prior to the data being unblinded.

To address our primary hypothesis, the analysis plan required two independent assessments of the responses in the three randomised arms of the study. First, to confirm our previous findings, we assessed whether the inhalation of diesel engine exhaust impaired vascular function and promoted thrombogenesis. Second, we assessed whether the particle trap improved these surrogate measures of cardiovascular health. Comparisons between exposures were undertaken using a two-sided paired *t* test and two-way ANOVA with repeated measures, as appropriate. Factors assessed in the two-way ANOVA were exposure and vasodilator dose. Exposure data were analysed using a two-sided unpaired *t* test. Statistical significance was taken at $P<0.05$.

7.4 RESULTS

The nineteen healthy male volunteers were young, normotensive and had normal lung function (Table 7.1). All volunteers completed the three study visits. Exposures were well tolerated with no adverse symptoms reported.

Age (years)	25 ± 3
Height (cm)	181 ± 5
Weight (kg)	75 ± 8
BMI (kg/m²)	23.4 ± 2
Pulse (bpm)	54 ± 8
Systolic Blood Pressure (mmHg)	113 ± 6
Diastolic Blood Pressure (mmHg)	73 ± 8
FEV_1 (L)	4.6 ± 0.6
% predicted FEV_1	100 ± 12
FVC (L)	5.6 ± 0.8
% predicted FVC	103 ± 14

Data shown are mean±standard error of the mean (n=19)

BMI = Body mass index
FEV_1 = Forced expiratory volume in one second
FVC = Forced vital capacity

Table 7.1 *Baseline subject characteristics (n=19)*

The particle trap reduced the total particle mass concentration in the chamber by approximately 98% and the fine (<1 μm) particle number concentration by more than 99.8%. As anticipated, the particle trap, with an integrated oxidation catalyst, did alter the composition of nitrogen oxides, i.e. increased NO_2 and decreased NO (Table 7.2). However, it had no significant effect on the concentrations of gaseous hydrocarbons.

There were no changes in blood pressure, resting heart rate, baseline forearm blood flow or markers of arterial stiffness in between the three study visits (data supplement, Tables 7b and 7c, below). Haematological variables, markers of inflammation and soluble markers of platelet activation did not differ between exposures (data supplement, Tables 7d and 7e, below). Markers of airway inflammation did not differ between exposures (data supplement, Table 7f, below).

	Unit	*Filtered diesel exhaust*	*Diesel exhaust*	*P-value*
NO	ppm	2.09 ± 0.15	5.72 ± 0.33	<0.001
NO_2	ppm	3.44 ± 0.33	0.69 ± 0.02	<0.001
NO_x	ppm	5.53 ± 0.44	6.40 ± 0.34	0.049
Total gaseous hydrocarbons	ppm	0.84 ± 0.06	0.91 ± 0.05	0.387
Total PM mass concentration	$\mu g/m^3$	7.2 ± 2.0	320 ± 10	<0.001
Fine particle number concentration	$\#/cm^3$	30 - 300	150,000 - 200,000	<0.001

Data shown are mean±standard error of the mean (n=19)
Data analysed using 2-tailed unpaired *t* test

NO - Nitric oxide, NO_2 - Nitrogen dioxide, NO_x - Nitrogen oxides, PM - Particulate matter

Table 7.2 *Exposure conditions in the chamber.*

7.4.1 VASCULAR STUDIES

Vasomotor Function

There was a dose-dependent increase in forearm blood flow with both endothelium-dependent (bradykinin and acetylcholine) and endothelium-independent (sodium nitroprusside and verapamil) vasodilators following each exposure (P<0.0001 for all; Figure 7.2).

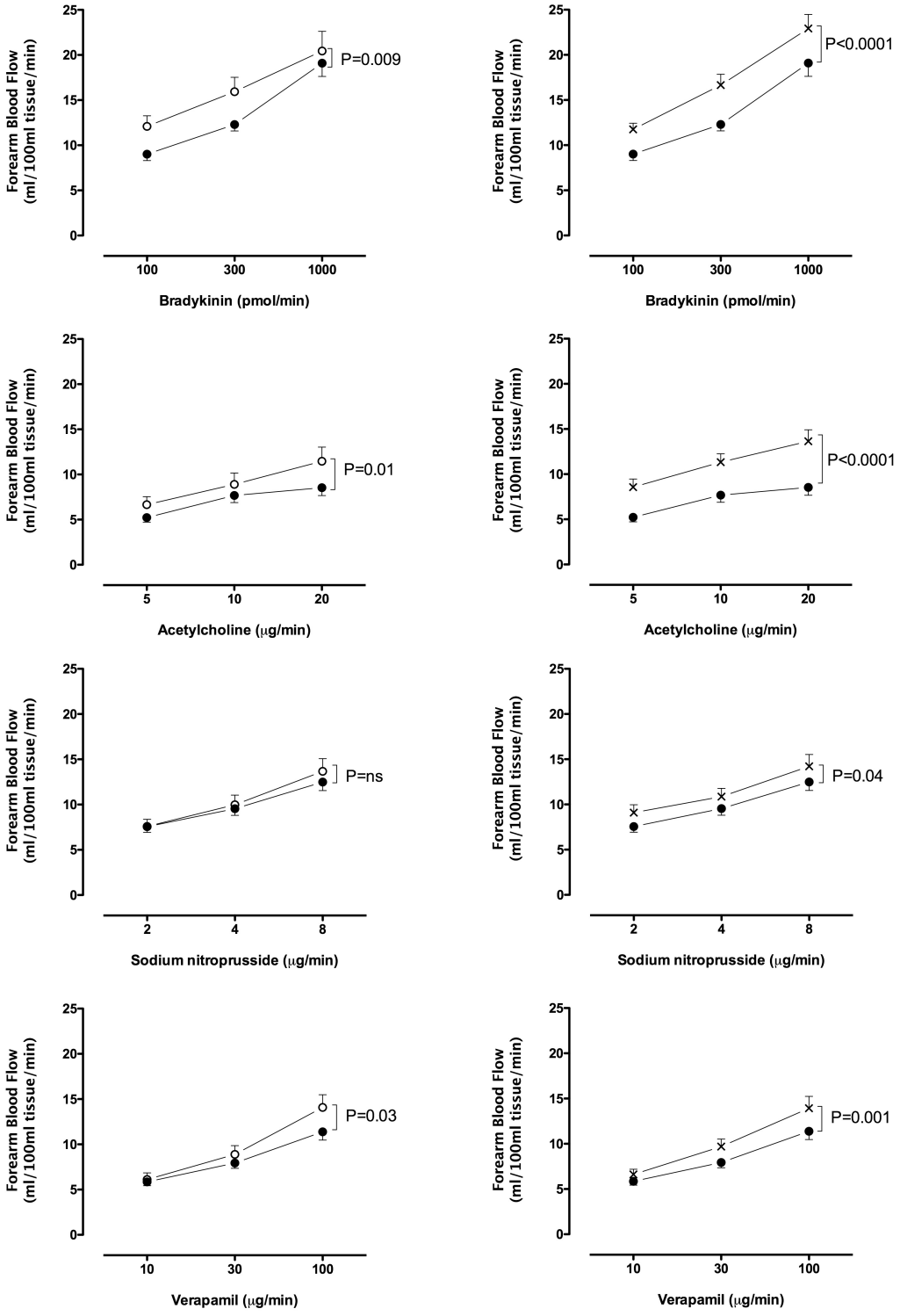

Figure 7.2 *Infused forearm blood flow four to six hours after exposure, during intra-brachial infusion of bradykinin, acetylcholine, sodium nitroprusside and verapamil. The left panel displays vasomotor response following exposure to air and diesel exhaust, confirming the vascular effects from previous investigations. Filtered air exposure shown by open circles, diesel engine exhaust exposure by filled circles. The right panel displays the main comparison of vasomotor function after exposure to unfiltered diesel exhaust (filled circles) and filtered diesel exhaust (crosses).*

Compared to filtered air, vasodilatation was impaired following diesel engine exhaust exposure in response to bradykinin (P=0.009), acetylcholine (P=0.01) and verapamil (P=0.03; Figure 7.2). There was no significant difference in response to sodium nitroprusside (P=0.15; Figure 7.2). However, with the introduction of the particle trap, vasodilatation increased to all vasodilators: bradykinin (P<0.0001), acetylcholine (P<0.0001), verapamil (P=0.001) and sodium nitroprusside (P=0.04; Figure 7.2). Indeed, there were no differences in vasomotor responses between filtered air and filtered diesel engine exhaust except for acetylcholine, where vasodilatation was lower with filtered air (P=0.02).

Fibrinolytic Function

There were no differences in baseline plasma t-PA and PAI-1 concentrations between exposures (data supplement, Table 7g, below). There was a dose-dependent increase in t-PA release in response to bradykinin infusion following each exposure (P<0.0001 for all; Figure 7.3). Although numerically lower, there was no statistical difference in t-PA-release following exposure to diesel engine exhaust inhalation compared with filtered air (P=0.30; Figure 7.3). However, application of the particle trap was associated with an improvement in the net release of t-PA compared to unfiltered diesel engine exhaust (P=0.03; Figure 7.3). There was no difference in t-PA-release between filtered air and filtered diesel engine exhaust (P=0.22).

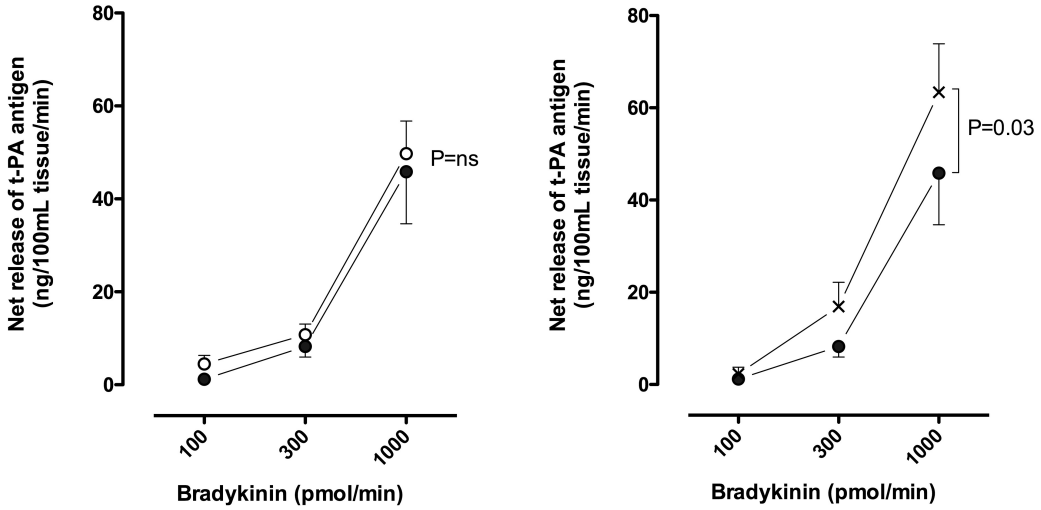

Figure 7.3 *Tissue plasminogen activator release from the forearm endothelium four to six hours after exposure, during intra-brachial infusion of bradykinin. The left panel displays fibrinolytic response following exposure to air and diesel exhaust. Filtered air exposure shown by open circles, diesel engine exhaust exposure by filled circles. The right panel displays the main comparison of fibrinolytic function after exposure to unfiltered diesel exhaust (filled circles) and filtered diesel exhaust (crosses).*

7.4.2 *EX VIVO* THROMBOSIS

Compared to filtered air, inhalation of diesel exhaust was associated with an increase of thrombus formation in the low-shear (21.8%, P<0.001; Figure 7.4) and high-shear (14.8%; P=0.02; Figure 7.4) chambers. Compared to unfiltered exhaust, the introduction of the particle trap was associated with a reduction in thrombus formation in the low-shear chamber (-15.7%, P=0.02; Figure 7.4), whilst the apparent reduction in the high-shear chamber did not reach statistical significance (P=0.11; Figure 7.4). There were no differences in thrombus formation between filtered air and filtered diesel exhaust (P=0.78 and P=0.76 for the low- and high-shear chambers respectively).

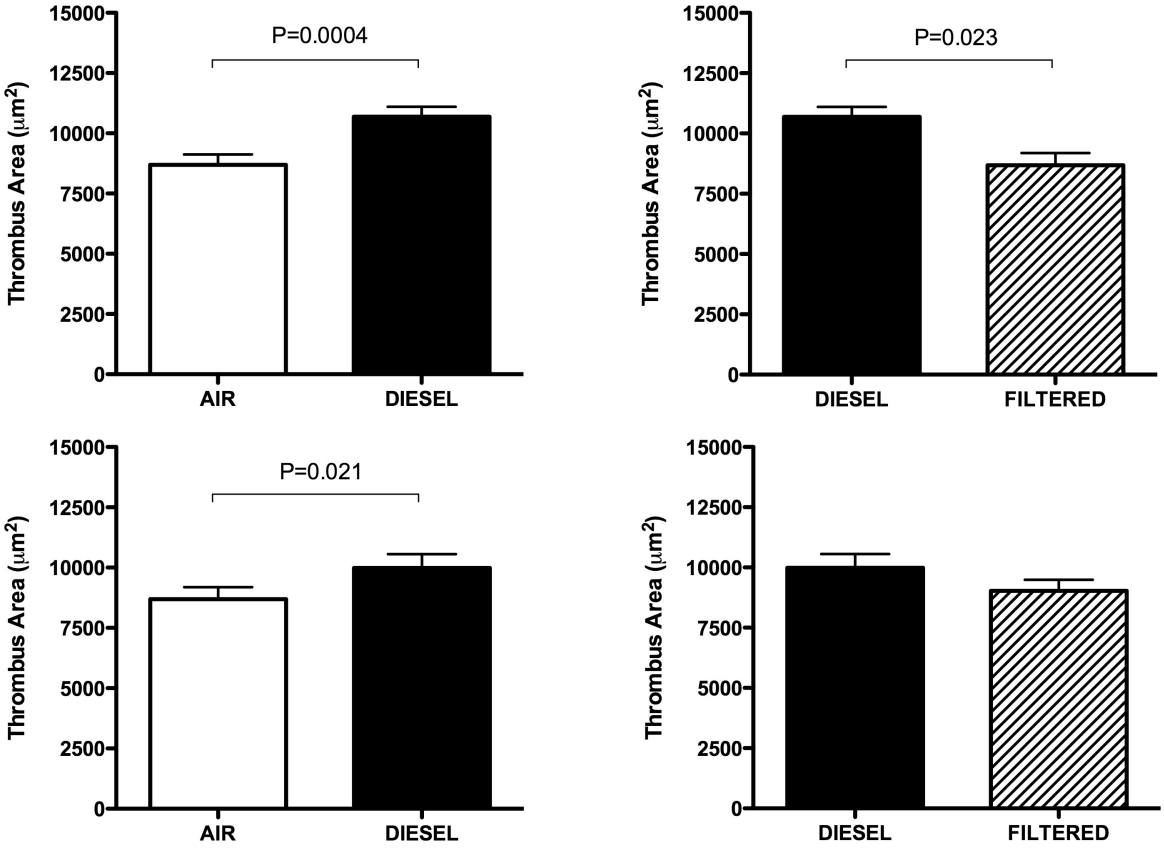

Figure 7.4 Ex vivo *thrombus formation assessed using the Badimon Chamber at two hours after exposure. Data from the low shear chamber are shown in the upper panels. Data from the high shear chamber are shown in the lower panels. Filtered air exposure shown in white, diesel engine exhaust exposure in black and filtered diesel exposure in hatched bars.*

7.5 DISCUSSION

Short-term exposure to traffic-derived air pollution is associated with acute cardiovascular events [Bhaskaran*, et al.*, 2009; Peters*, et al.*, 2001; Peters*, et al.*, 2004]. In the current study, using complementary and relevant measures of cardiovascular health, we have reconfirmed the adverse effects of exposure to diesel engine exhaust on endothelial function and *ex vivo* thrombosis. In addition, for the first time, we demonstrate that reducing the particulate component of diesel exhaust using a commercially available particle trap can prevent these detrimental cardiovascular effects. Our study provides support for the application of particle traps to diesel-powered vehicles in order to reduce urban particulate concentrations and limit a range of adverse cardiovascular effects of exposure to traffic-derived air pollution.

In a series of controlled exposure studies in human subjects, we have previously shown an impairment of vasomotor responses to endothelium-dependent and endothelium-independent vasodilators after diesel exhaust exposure [Tornqvist*, et al.*, 2007]. These observations are consistent with other reports of brachial artery vasoconstriction shortly after exposure to dilute diesel exhaust [Peretz*, et al.*, 2008] and concentrated ambient particles [Brook*, et al.*, 2002]. Such vascular impairment is not restricted to vasomotor function. We have also demonstrated increased thrombogenicity with reduced t-PA release from the endothelium [Mills*, et al.*, 2005], enhanced platelet activation [Lucking*, et al.*, 2008] and increased *ex vivo* thrombus formation [Lucking*, et al.*, 2008].

In the present study we employed a range of these complementary measures of cardiovascular function in a comprehensive assessment of the potential for particle traps to improve human health. We were able to confirm our earlier findings that diesel exhaust inhalation causes detrimental vascular and prothrombotic effects. On this occasion, we did not observe a statistically significant reduction in t-PA release from the endothelium following diesel exhaust exposure and this may represent a type II error or reflect the subtle differences between study protocols. However, more importantly we were able to demonstrate that the introduction of a particle trap not only improved vasomotion, endogenous fibrinolysis and *ex vivo* thrombosis but appeared to normalise them.

Although there are many potentially harmful components in ambient air pollution, traffic-derived fine and ultra-fine particles are most closely and consistently linked to acute cardiovascular events. This has been the rationale for the development, and legislation for, targeted interventions to reduce the particulate matter content of vehicle emissions. Whilst there is little doubt that particle traps are effective in reducing PM mass and number, concerns have been raised regarding the oxidation catalysts required to regenerate and maintain filter efficiency as they may alter the toxicity of particulate and gaseous emissions. For example, soot particles generated from a low emission diesel engine appear to have greater cytotoxic and pro-inflammatory effects [Su*, et al.*, 2008]. The potential for particle traps to reduce the adverse cardiovascular effects of diesel exhaust emissions therefore needs to be assessed in man. Here we observed no adverse cardiovascular effects arising from the use of a particle trap. The only difference between filtered air and filtered diesel exhaust observed for any variable we assessed was, in fact, an enhancement of

vasodilatation in response to acetylcholine following exposure to filtered diesel exhaust. Whilst this difference was statistically significant, it was numerically small and unlikely to be of major physiological significance. In light of these factors, we suspect that this may be a result of a type I error although we cannot rule out an effect related to alterations in one or more unmeasured gaseous components.

We employed a commercially available particle trap to reduce particle emissions from a heavy-duty diesel engine operating under the transient cycling conditions used as the standard for engine testing across the European Union. Particle filtration markedly reduced the mass and number of particle emissions. Taken together with our previous findings and data from observational studies, we believe it is this reduction that is responsible for rectifying the adverse cardiovascular effects of diesel engine exhaust inhalation. As expected, oxidation catalysts within the particle trap altered the composition of NO_x with an increase in NO_2 and decrease in NO concentrations. However, we have recently assessed the effects of NO_2 on healthy volunteers and did not identify any adverse effects on vascular or fibrinolytic function [Langrish*, et al.*]. Whilst we did not observe an effect on the other gaseous components of the exposure, we acknowledge that the particle trap may have altered the composition of the exposures beyond those variables assessed during this study.

Consistent with previous studies [Barath*, et al.*; Mills*, et al.*, 2005; Tornqvist*, et al.*, 2007] vasodilatation was impaired following exposure to diesel engine exhaust in response to bradykinin and acetylcholine. However, there was also a reduction in vasodilatation to verapamil, implying an additional calcium flux-dependent impairment of vascular smooth muscle function. A reduction in vasodilatation to

verapamil has previously been demonstrated following exposure to diesel exhaust generated under transient engine speed and load [Barath*, et al.*] but not when generated under idling conditions [Mills*, et al.*, 2005]. We have previously speculated that, in exhaust generated under transient running conditions, the higher diesel-related soot content and its associated (adsorbed) organic material may cause these additional vascular smooth muscle effects and believe that this observation warrants further investigation [Barath*, et al.*]. Interestingly, impaired vasodilatation in response to verapamil was also normalised by the introduction of a particle trap. Although variations in responses between studies with different exposure protocols might provide insights into the pathophysiological mechanisms responsible for the adverse vasomotor effects of diesel exhaust exposure, these studies were designed and powered to detect differences within rather than between studies. Thus, whilst tempting, we believe that we should be cautious and circumspect in drawing conclusions from comparisons made between studies and any such differences should be regarded as hypothesis generating and the subject of future investigations.

The exact mechanisms underlying the vascular and prothrombotic effects we observed in the current and previous studies remain only partially understood. Regarding the increase in *ex vivo* thrombosis seen following diesel exhaust exposure, data from previous *in vitro* [Radomski, *et al.,* 2005] and animal studies [Nemmar*, et al.*, 2003], as well as our own previous controlled exposure studies in humans [Lucking*, et al.*, 2008], suggest that platelet activation plays a central role. Platelets are key components of arterial thrombosis, a process that underpins acute coronary syndromes including myocardial infarction. Platelet-leucocyte aggregates, increasingly recognised as the gold standard measure of *in vivo* platelet activation [Freedman*, et*

al., 2002], were increased following tracheal instillation of carbon nanotubes in a murine model of vascular injury [Nemmar*, et al.*, 2007] and following diesel exhaust exposure in humans [Lucking*, et al.*, 2008]. Debate remains as to whether inhaled components of diesel exhaust can translocate into the systemic circulation to mediate direct effects on blood and vascular components [Mills*, et al.*, 2006; Nemmar*, et al.*, 2002] or whether the induction of pulmonary inflammation and the subsequent generation of free radicals may activate platelets by reducing endothelium- and platelet-derived nitric oxide and antioxidants. Although a single observational study reported a small reduction in prothrombin time associated with ambient exposure to PM_{10}, [Baccarelli*, et al.*, 2007] the authors are not aware of any controlled exposure study demonstrating an effect of pollution exposure on plasma concentrations of coagulation factors.

The potential mechanisms underlying the adverse vasomotor effects observed in response to diesel exhaust exposure remain only partly understood [Brook*, et al.*] and a full discussion is beyond the scope of this manuscript. However, based on data from our earlier studies in which the exposure was generated by an idling diesel engine [Mills*, et al.*, 2005; Tornqvist*, et al.*, 2007], we have previously speculated that oxidative stress and impaired NO-dependent signalling play a central role in the adverse vasomotor effects. Given the broader impairment of vasomotor function we observed here and in a previous study using a transient cycling diesel engine [Barath*, et al.*], we acknowledge that we cannot discount upregulation of other circulating or cellular vasoconstrictor mediators (such as Rho Kinase [Sun*, et al.*, 2008]) or activation of the sympathetic nervous system as an alternative explanation for the general blunting of vasodilator responses observed.

7.5.1 LIMITATIONS

Our principal objective was to assess the impact of a commercially available particle trap on markers of cardiovascular health by comparing the effects of unfiltered and filtered diesel exhaust. In this regard, we clearly demonstrate that vasodilatation, endothelial t-PA release and thrombus formation are improved by particle filtration. However, we acknowledge that our approach has limitations. The use of multiple and complementary surrogates of cardiovascular health is both a strength and a weakness of this study. Replication of previous observations suggests that the findings are real and provides a clear and consistent message; diesel exhaust impairs vascular function [Barath*, et al.*; Mills*, et al.*, 2005; Tornqvist*, et al.*, 2007] and increases *ex vivo* thrombus formation [Lucking*, et al.*, 2008]. The use of multiple end-points with three exposure conditions requires multiple comparisons and increases the possibility of type I and II errors. We have not adjusted for multiple comparisons as the initial comparison between diesel exhaust and filtered air was made simply to confirm our previous findings. Both the direction and magnitude of the changes seen here are consistent with our previous findings although the differences in net t-PA release failed to achieve statistical significance. Although the study was powered prospectively based on measurements of the primary endpoints made during previous diesel exposure studies, we acknowledge that the sample size is modest. Whilst we are confident that we have not missed effects on endothelial function or *ex vivo* thrombosis, we acknowledge that we may have insufficient power to detect changes in some of the secondary endpoints (data supplement, Table 1) and thus cannot exclude the possibility of false negative findings confounding their assessment. In addition, the study cohort consisted exclusively of young, healthy men. Although one might postulate that the benefit of particle traps may actually be greater in those with

pre-existing cardiovascular disease, we concede that further studies are required to assess the role of particle traps in mitigating cardiovascular effects in women and the broader population.

7.5.2 CONCLUSION

Using several surrogate measures, a range of adverse cardiovascular effects of diesel exhaust inhalation in man appears to be prevented by the introduction of a particle trap. Given these beneficial effects on biomarkers of cardiovascular health, the widespread use of particle traps on diesel-powered vehicles may have substantial public health benefit and reduce the burden of cardiovascular disease.

7.6 SUPPLEMENTAL MATERIAL (published as online supplement)

7.6.1 SUPPLEMENTAL METHODS

Power Calculations

The studies were prospectively powered based on measurements of the primary endpoints (endothelial vasomotor function and endogenous fibrinolysis assessed by forearm venous occlusion plethysmography; and *ex vivo* thrombus formation assessed using the Badimon Chamber). Based on our previous studies of endothelial vasomotor function and endogenous fibrinolysis, to detect a 20% difference in forearm blood flow and a 16% difference in t-PA release, we require sample sizes of n=18 at 90% power and two-sided $P<0.05$. Based on previous studies by our own and Professor Badimon's group, to detect differences of 10% in thrombus area, we require sample sizes of n=18 at 90% power and two-sided $P<0.05$. Power calculations for the secondary and other endpoints are presented in Table 7a.

Arterial Stiffness

All measurements were performed by a single operator who was unaware of the nature of exposure. Studies were performed in a quiet, temperature controlled room with subjects resting in a supine position. Systolic and diastolic blood pressures were measured using a semi automated non-invasive oscillometric sphygmomanometer (Omron HEM-705CP, Omron, Matsusaka, Japan). Pulse wave analysis was performed using applanation tonometry (Millar Instruments, Texas, USA) of the radial artery and the SphygmoCor™ system (AtCor Medical, Sydney, Australia) in accordance with the manufacturer's recommendations. Briefly, pulse wave analysis derives an aortic pulse pressure waveform from the radial artery wave via a matheamatical transfer function. The arterial pressure waveform is a composite of the

forward pressure wave created by ventricular contraction and a reflected wave generated by peripheral vascular resistance. The augmentation pressure is the difference between the second and first systolic peaks. The augmentation index (augmentation pressure as a percentage of the pulse pressure) is a measure of systemic arterial stiffness and wave reflection. The time to wave reflection is reduced with increasing arterial stiffness, and provides a surrogate of aortic pulse wave velocity [Mills*, et al.*, 2008]. At least two independent waveform analyses were obtained from each subject, with measurements only accepted upon meeting SphygmoCor™ quality control criteria. Pulse wave velocity was calculated by measuring the time for the pulse wave to travel between the carotid and femoral arteries.

Fraction of Exhaled Nitric Oxide

All measurements were performed by a single blinded operator. Fraction of exhaled nitric oxide (FE_{NO}) concentrations were evaluated pre exposure, 2 and 6 hours post-exposure, using a nitric oxide analyser (NIOX®, Aerocrine AB, Stockholm, Sweden). Two different exhalation flow rates; 10 and 50 $mL \times s^{-1}$ (± 10%), during a slow exhalation against an oral pressure of 2 cm H_2O for 8 seconds were examined. The measurements were conducted in triplicate and the mean concentration of exhaled NO (ppb) was registered according to current ERS/ATS guidelines.

Data Analysis and Statistics

All studies, data analysis and data exclusion were performed prior to the data being unblinded. Continuous variables are reported as mean ± standard error of the mean (SEM). Statistical analyses were performed with GraphPad Prism (Graph Pad Software, California, USA).

Comparisons between exposures were undertaken using one- and two-way analysis of variance (ANOVA) with repeated measures, as appropriate. Factors assessed in the two-way ANOVA were exposure and time. Statistical significance was taken at $P<0.05$.

7.6.2 SUPPLEMENTAL RESULTS

There were no differences in resting heart rate, blood pressure or arterial stiffness following exposure to diesel exhaust, filtered exhaust or filtered air (Tables b and c). Haematological variables, plasma markers of inflammation and platelet activation, baseline markers of fibrinolytic function and markers of airway inflammation were not different between exposures (Tables d-g).

7.6.3 SUPPLEMENTAL FIGURE

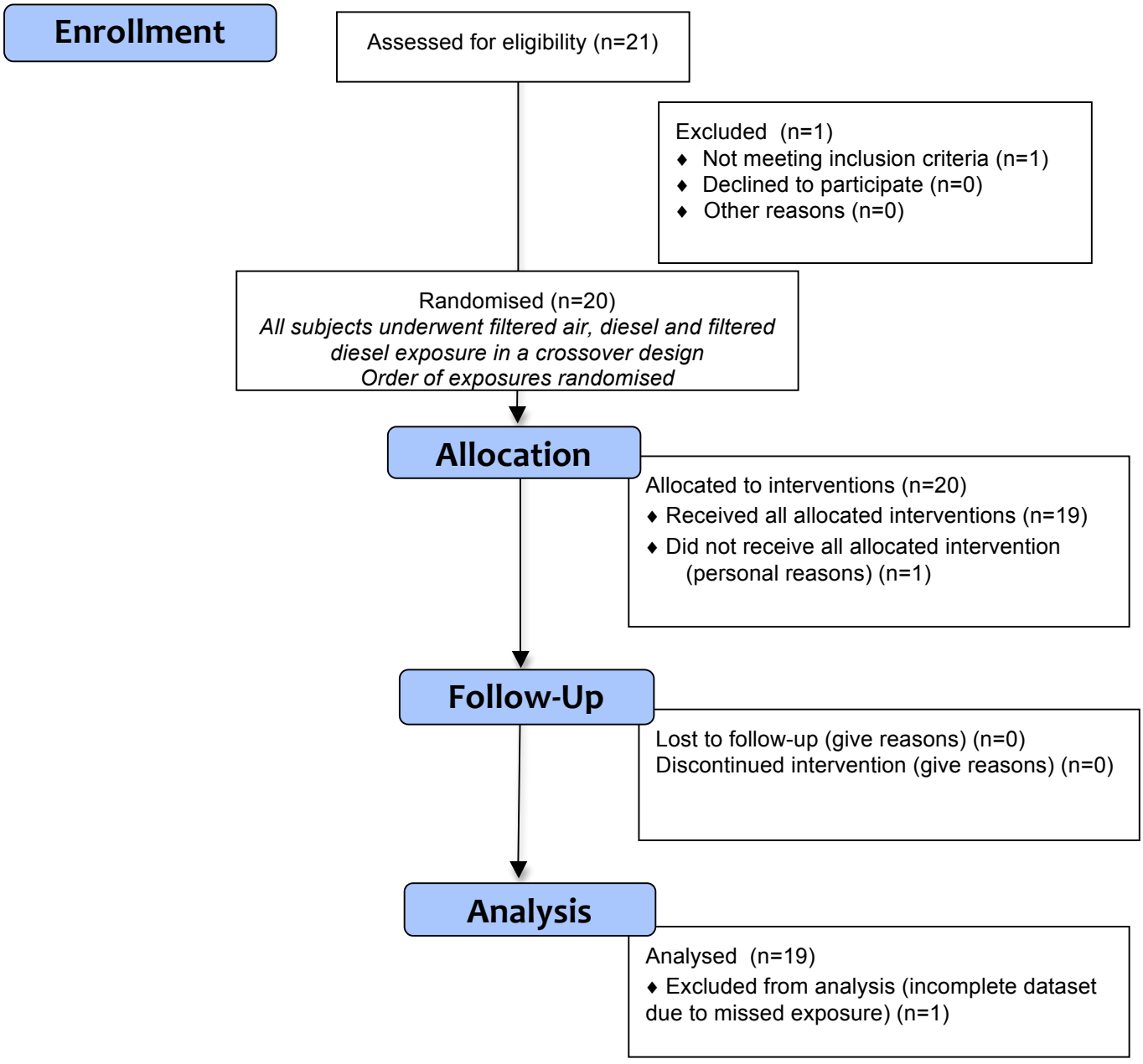

Figure 7a *CONSORT diagram*

7.6.4 SUPPLEMENTAL TABLES

	n	mean	SD	SD^2	*Effect size detectable at:* 80% power	90% power
Systolic blood pressure (mmHg)	19	136	14	190	13	14
Diastolic blood pressure (mmHg)	19	70	9.9	100	9.0	10
Pulse (bpm)	19	64	12	132	10	12
TNF-a (pg/mL)	19	0.63	0.31	0.10	0.28	0.32
IL-6 (pg/mL)	19	0.28	0.21	0.04	0.19	0.22
CRP (mg/mL)	19	0.56	0.28	0.08	0.25	0.29
Soluble CD40L (pg/mL)	19	61	18	339	17	19
Soluble P-selectin (mg/mL)	19	39	11	110	9.5	11
Soluble ICAM-1 (mg/mL)	19	231	41	1658	37	43
Baseline t-PA (ng/mL)	19	5.0	3.9	15	3.5	4.0
Baseline PAI-1 (ng/mL)	19	6.3	3.8	14	3.4	4.0
Augmentation Pressure (mmHg)	19	-0.70	3.9	15	3.5	4.1
Augmentation Index (%)	19	-2.6	12	142	11	12
Time to Wave reflection (ms)	19	179	28	784	25	29
FE_{NO} 50 (ppb)	19	13	5.2	27	4.7	5.4
FE_{NO} 10 (ppb)	19	44	19	375	18	20

Table a *Power calculations for the secondary endpoints*

	Before Exposure	*After Exposure*	*6 Hours*	*8 Hours*
FILTERED AIR				
Systolic Blood Pressure (mmHg)	135 ± 3	139 ± 3	141 ± 3	139 ± 3
Diastolic Blood Pressure (mmHg)	73 ± 3	66 ± 2	66 ± 2	71 ± 3
Pulse (bpm)	73 ± 3	64 ± 2	63 ± 2	59 ± 3
Infused FBF (mL/100mL tissue/min)	-	-	2.1 ± 0.1	-
Noninfused FBF (mL/100mL tissue/min)	-	-	2.0 ± 0.1	-
DIESEL EXHAUST				
Systolic Blood Pressure (mmHg)	133 ± 3	131 ± 3	132 ± 3	135 ± 3
Diastolic Blood Pressure (mmHg)	75 ± 2	66 ± 2	66 ± 2	69 ± 2
Pulse (bpm)	72 ± 2	63 ± 3	59 ± 2	57 ± 2
Infused FBF (mL/100mL tissue/min)	-	-	2.1 ± 0.1	-
Noninfused FBF (mL/100mL tissue/min)	-	-	2.1 ± 0.2	-
FILTERED DIESEL EXHAUST				
Systolic Blood Pressure (mmHg)	132 ± 3	137 ± 3	138 ± 4	137 ± 3
Diastolic Blood Pressure (mmHg)	77 ± 2	70 ± 2	69 ± 3	72 ± 2
Pulse (bpm)	74 ± 2	63 ± 2	59 ± 2	59 ± 2
Infused FBF (mL/100mL tissue/min)	-	-	2.1 ± 0.1	-
Noninfused FBF (mL/100mL tissue/min)	-	-	2.1 ± 0.2	-

Data shown are mean±standard error of the mean (n=19)

There were no significant differences between exposures (2 way ANOVA with repeated measures)

FBF - Forearm blood flow

Table b *Haemodynamic variables*

Time after exposure	*+5 mins*	*+20 mins*	*+30 mins*	*+50mins*
FILTERED AIR				
Augmentation Pressure (mmHg)	0.9 ± 1.4	0.0 ± 1.1	-1.1 ± 0.8	0.1 ± 1.0
Augmentation Index (%)	2.0 ± 3.8	-0.8 ± 3.3	-4.7 ± 2.5	-0.2 ± 3.1
Time to Wave Reflection (ms)	178 ± 10	175 ± 8	184 ± 7	165 ± 7
Systolic Blood Pressure (mmHg)	141 ± 3	137± 2	139 ± 3	137 ± 3
Diastolic Blood Pressure (mmHg)	71 ± 2	69 ± 3	66 ± 3	69 ± 3
Pulse (bpm)	63 ± 3	63 ± 3	62 ± 2	63 ± 2
DIESEL EXHAUST				
Augmentation Pressure (mmHg)	-0.6 ± 0.7	-0.6 ± 0.9	-0.8 ± 0.9	-0.6 ± 1.1
Augmentation Index (%)	-1.8 ± 2.2	-2.3 ± 2.9	-2.7 ± 2.9	-2.9 ± 3.7
Time to Wave Reflection (ms)	174 ± 8	188 ± 7	180 ± 7	185 ± 8
Systolic Blood Pressure (mmHg)	136 ± 3	138 ± 3	142 ± 4	138 ± 3
Diastolic Blood Pressure (mmHg)	69 ± 3	70 ± 2	70 ± 2	67 ± 2
Pulse (bpm)	58 ± 2	58 ± 3	59 ± 2	59 ± 2
FILTERED DIESEL EXHAUST				
Augmentation Pressure (mmHg)	-0.9 ± 0.9	-1.9 ± 1.0	-2.1 ± 1.0	-1.5 ± 1.1
Augmentation Index (%)	-2.5 ± 2.9	-7.1 ± 3.2	-7.1 ± 3.0	-5.9 ± 3.3
Time to Wave Reflection (ms)	177 ± 8	183 ± 7	181 ± 7	186 ± 7
Systolic Blood Pressure (mmHg)	144 ± 3	139 ± 3	140 ± 2	140 ± 3
Diastolic Blood Pressure (mmHg)	74 ± 2	70 ± 2	74 ± 3	72 ± 2
Pulse (bpm)	63 ± 2	60 ± 3	61 ± 2	62 ± 2

	Pulse wave velocity, m/s	
Time after exposure	***+10 mins***	***+40 mins***
Filtered air	5.6 ± 0.4	5.2 ± 0.1
Diesel exhaust	5.0 ± 0.1	5.0 ± 0.1
Filtered diesel exhaust	4.9 ± 0.1	5.0 ± 0.1

Data shown are mean±standard error of the mean (n=19)

There were no significant differences between exposures (2 way ANOVA with repeated measures)

Table c *Pulse wave analysis and pulse wave velocity*

	Before Exposure	*2 Hours*	*6 Hours*	*8 Hours*
FILTERED AIR				
Haemoglobin (g/L)	143±2	137±2	137±2	135±2
Leucocytes ($\times 10^9$ cells/L)	4.9±0.3	5.2±0.4	5.1±0.4	5.3±0.4
Lymphocytes ($\times 10^9$ cells/L)	1.8±0.1	1.7±0.1	1.6±0.1	1.8±0.1
Neutrophils ($\times 10^9$ cells/L)	2.4±0.2	2.9±0.3	2.9±0.3	2.8±0.3
Monocytes ($\times 10^9$ cells/L)	0.5±0.0	0.5±0.0	0.4±0.0	0.5±0.0
Platelets ($\times 10^9$ cells/L)	202±11	203±10	205±11	201±9
DIESEL EXHAUST				
Haemoglobin (g/L)	141±2	137±2	135±2	134±2
Leucocytes ($\times 10^9$ cells/L)	5.0±0.3	5.3±0.3	5.2±0.3	5.5±0.3
Lymphocytes ($\times 10^9$ cells/L)	1.8±0.1	1.8±0.1	1.7±0.1	1.9±0.1
Neutrophils ($\times 10^9$ cells/L)	2.4±0.2	2.9±0.2	2.9±0.2	2.9±0.2
Monocytes ($\times 10^9$ cells/L)	0.5±0.0	0.5±0.0	0.4±0.0	0.5±0.0
Platelets ($\times 10^9$ cells/L)	201±9	204±10	207±9	201±9
FILTERED DIESEL EXHAUST				
Haemoglobin (g/L)	141±2	137±2	135±2	133±2
Leucocytes ($\times 10^9$ cells/L)	4.9±0.2	5.2±0.3	5.1±0.2	5.3±0.2
Lymphocytes ($\times 10^9$ cells/L)	1.9±0.1	1.8±0.1	1.7±0.1	1.9±0.1
Neutrophils ($\times 10^9$ cells/L)	2.3±0.1	2.8±0.2	2.9±0.2	2.7±0.2
Monocytes ($\times 10^9$ cells/L)	0.5±0.0	0.4±0.0	0.4±0.0	0.5±0.0
Platelets ($\times 10^9$ cells/L)	200±8	190±8	201±8	194±8

Data shown are mean±standard error of the mean (n=19)

There were no significant differences between exposures (2 way ANOVA with repeated measures)

Table d *Haematological variables*

	Before Exposure	*2 Hours*	*6 Hours*
FILTERED AIR			
TNF-α (pg/mL)	0.59±0.07	0.63±0.08	0.65±0.06
IL-6 (pg/mL)	0.29±0.05	0.32±0.06	0.23±0.04
CRP (mg/L)	0.55±0.06	0.55±0.06	0.58±0.07
Soluble CD40L (pg/mL)	62±2	57±3	63±7
Soluble P-selectin (mg/mL)	40±3	40±2	37±2
Soluble ICAM-1 (mg/mL)	228±10	241±8	223±11
DIESEL EXHAUST			
TNF-α (pg/mL)	0.61±0.06	0.65±0.07	0.65±0.06
IL-6 (pg/mL)	0.35±0.08	0.40±0.08	0.31±0.08
CRP (mg/L)	0.60±0.11	0.52±0.08	0.56±0.07
Soluble CD40L (pg/mL)	67±3	60±2	60±2
Soluble P-selectin (mg/mL)	42±2	38±3	40±2
Soluble ICAM-1 (mg/mL)	254±10	220±13	234±10
FILTERED DIESEL EXHAUST			
TNF-α (pg/mL)	0.61±0.06	0.58±0.07	0.61±0.07
IL-6 (pg/mL)	0.33±0.06	0.33±0.07	0.36±0.07
CRP (mg/L)	0.61±0.10	0.60±0.08	0.64±0.10
Soluble CD40L (pg/mL)	63±4	61±4	62±4
Soluble P-selectin (mg/mL)	39±2	39±2	39±2
Soluble ICAM-1 (mg/mL)	242±11	243±14	238±10

Data shown are mean±standard error of the mean (n=19)

There were no significant differences between exposures (2 way ANOVA with repeated measures)

TNF-α - Tumour necrosis factor-α, IL-6 - Interleukin-6, CRP - C reactive protein, CD40L - CD40 ligand, ICAM-1 - Intercellular adhesion molecule-1

Table e *Markers of inflammation and platelet activation*

	Before Exposure	*2 Hours*	*6 Hours*
FILTERED AIR			
FE_{NO} 50 (ppb)	13 ± 1.2	16 ± 2.1	16 ±2.3
FE_{NO} 10 (ppb)	46 ± 4.9	51 ± 8.6	54 ± 8.1
DIESEL EXHAUST			
FE_{NO} 50 (ppb)	13 ± 1.2	14 ± 1.3	15 ± 2.1
FE_{NO} 10 (ppb)	44 ± 4.4	47 ± 4.7	48 ± 7.1
FILTERED DIESEL EXHAUST			
FE_{NO} 50 (ppb)	12 ± 0.9	13 ± 1.1	13 ± 1.0
FE_{NO} 10 (ppb)	41 ± 3.3	45 ± 3.3	44 ± 3.6

Data shown are mean±standard error of the mean (n=19)

There were no significant differences between exposures (2 way ANOVA with repeated measures)

FE_{NO} 50 - Fraction of exhaled nitric oxide at exhalation rate 50 mL/s, FE_{NO} 10 - Fraction of exhaled nitric oxide at exhalation rate 10 mL/s

Table f *Markers of airway inflammation*

	Six Hours
FILTERED AIR	
Tissue plasminogen antigen (ng/mL)	2.9 ± 0.5
Plasminogen activator inhibitor type 1 (ng/mL)	4.8 ± 0.5
DIESEL EXHAUST	
Tissue plasminogen antigen (ng/mL)	2.9 ± 0.5
Plasminogen activator inhibitor type 1 (ng/mL)	5.4 ± 0.6
FILTERED DIESEL EXHAUST	
Tissue plasminogen antigen (ng/mL)	3.4 ± 0.6
Plasminogen activator inhibitor type 1 (ng/mL)	5.1 ± 0.5

Data shown are mean±standard error of the mean (n=19)

There were no significant differences between exposures (1 way ANOVA with repeated measures)

Table g *Markers of fibrinolytic function*

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

8.1 SUMMARY OF FINDINGS

Cardiovascular disease remains the commonest cause of premature death in the developed world and its incidence is rapidly increasing in developing countries. The dynamic regulation of intravascular thrombus formation is central to the pathogenesis of acute atherosclerotic events, particularly within the coronary circulation [Rosenberg*, et al.*, 1999]. Whilst several preclinical models of thrombosis exist, species-specific differences ensure that clinical studies remain an essential part of translational research and antithrombotic drug development. The demonstration of antithrombotic efficacy in man is challenging and an *in vivo* model for use in clinical studies does not currently exist. The Badimon chamber is an *ex vivo* model of thrombosis that is suitable for use in clinical studies and has previously been used principally to assess novel antithrombotic agents and regimens in patients undergoing percutaneous coronary intervention [Lev*, et al.*, 2006; Wahlander*, et al.*, 2006; Zafar*, et al.*, 2007]. Whilst it has the advantage of allowing assessment of thrombus formation on a pathophysiologically relevant substrate and under conditions of continuous flow, it has limitations that curtail its broader applicability. In addition, previous characterisation studies [Badimon*, et al.*, 1987] were performed in a porcine system using methodology that has since been superseded and there are no published data specifically addressing the reproducibility of thrombus formation in the chamber.

During this body of work I have sought to establish the Badimon Chamber technique within my institution and adapt aspects of the methodology in order to broaden its applicability and allow important clinical questions to be addressed. As such the work can be broadly divided into three phases.

The initial validation phase involved establishing the technique in my own institution and demonstrating it provided a robust method for assessing thrombus formation. As part of this initial phase, I adapted the existing methodology to allow the accurate addition of experimental agents and compounds to the extracorporeal circuit and modified the analysis phase to make use of contemporary semi-automated image acquisition and analysis equipment. The technique is now more efficient and can be used to safely assess novel compounds not previously tested in humans. As planned, these modifications together have significantly broadened its applicability.

In the subsequent phases, I have demonstrated this enhanced applicability by utilising the modifications made to the methodology to address important questions in two separate but overlapping areas of cardiovascular medicine. Specific findings are discussed below.

8.1.1 VALIDATION, CHARACTERISATION AND REPRODUCIBILITY

The initial studies, performed in healthy volunteers, provided important validation data confirming that the technique had been successfully established within our institution. Histological data demonstrated that the thrombus formed within the chamber is platelet-rich and forms upon a basal layer of fibrin. The contribution of fibrin was greater under conditions of lower shear stress, whilst platelets contributed more under conditions of high shear stress. Importantly, we demonstrated that total thrombus area is highly reproducible both within and between days, indicating that the technique provides a flexible model with which to assess novel and existing interventions using parallel group and crossover study designs.

8.1.2 ENDOGENOUS TISSUE PLASMINOGEN ACTIVATOR ENHANCES FIBRINOLYSIS

The second phase involved using the adapted methodology to establish a technique for assessing fibrinolysis. Previous work within our institution has provided novel insights into the role of endogenous fibrinolysis, endothelial function and intravascular thrombosis [Labinjoh, *et al*., 2001; Newby, *et al*., 2001; Newby, *et al*., 1997; Robinson, *et al*., 2007]. However, the primary end-point in all previous studies has been the quantification of t-PA release from the endothelium.

In the first part of this study, we demonstrated that the addition of exogenous t-PA into the extracorporeal circuit could be achieved in a reliable, predictable and reproducible manner allowing the demonstration of a dose dependent reduction in thrombus formation. The associated dose dependent increase in D-dimer levels observed in the chamber effluent is consistent with enhanced fibrinolysis and confirms that the technique can be used as a clinical model of fibrinolysis.

In the second part of the study, by combining intra-arterial infusion of bradykinin into the human forearm in order to stimulate acute release of endogenous t-PA with an assessment of dynamic thrombus formation using the Badimon Chamber, we demonstrated that t-PA released in this manner enhances fibrinolysis and is able to limit *in situ* thrombus formation.

These studies in combination confirm the functional significance of t-PA released during agonist stimulation, support a crucial role for the endogenous fibrinolytic system and suggest that further studies to explore its therapeutic value are warranted.

8.1.3 EVALUATING THE SMALL MOLECULE PAI-1 INHIBITOR, PAI-749

Having demonstrated that the technique can be adapted to investigate novel agents with the potential to enhance fibrinolysis, we went on to evaluate a promising small molecule PAI-1 inhibitor, PAI-749, in a double blind randomised controlled study. *Ex vivo* studies were complemented by extensive complementary *in vitro* assessments performed in collaboration with colleagues in Aberdeen and Leeds. Interestingly, in contrast to the promising results seen with this and other similar compounds in preclinical models [Gardell*, et al.*, 2007; J. Hennan, 2006], we were unable to demonstrate efficacy in any of the clinical models used. The reason(s) underlying the lack of efficacy was not entirely clear. Compared to previous preclinical studies, exposure time in our studies was significantly less and the models we employed were selected principally to assess fibrinolytic efficacy rather than to assess antithrombotic efficacy that had formed the basis of the preclinical studies. In addition to casting doubt on future development of this particular compound, the study highlights the potential pitfalls of relying solely on *in vitro* and pre-clinical models during early compound development. Indeed, by adding compounds to the extracorporeal circuit, we highlight a particular strength of the Badimon Chamber in that the efficacy of novel compounds can be evaluated in a robust and relevant clinical model without the subject being exposed. This may allow the more timely identification of compounds that show promise in preclinical models but, due to species differences, fail to demonstrate efficacy in man. The ongoing development of such compounds could then be discontinued earlier saving time and money.

8.1.4 DIESEL EXPOSURE INCREASES THROMBUS FORMATION IN MAN

Robust observational data suggest that acute exposure to particulate air pollution can trigger vascular events including acute myocardial infarction [Peters*, et al.*, 2001; Peters*, et al.*, 2004]. The biological mechanisms underlying this are only partly understood and may be multifactorial. Preclinical data suggest that platelet activation and thrombosis may be important [Nemmar*, et al.*, 2003]. In collaboration with colleagues from The University of Umeå, Sweden, 20 healthy men were exposed in a double blind randomised controlled study to dilute diesel exhaust or air under controlled conditions and according to a well-established protocol. This was the first study to demonstrate that inhalation of diesel exhaust, a common urban air pollutant, causes platelet activation and enhances thrombus formation in man. The data provide a plausible mechanism linking exposure to particulate air pollution with acute cardiovascular events including myocardial infarction.

8.1.5 PREVENTING THE ADVERSE EFFECTS OF DIESEL EXPOSURE

In this subsequent study, again conducted in conjunction with colleagues in Sweden and The Netherlands, we exposed 19 healthy men to dilute diesel exhaust, air or diesel exhaust filtered through a particle trap. Using a powerful study design, we demonstrated for the first time that reducing the particulate component of diesel exhaust using a commercially available particle trap can prevent the detrimental effects on *ex vivo* thrombosis and endothelial function. The study supports calls for the application of particle traps to diesel-powered vehicles in order to reduce urban particulate concentrations and limit a range of adverse cardiovascular effects that result from exposure to traffic-derived air pollution.

8.2 FUTURE DIRECTIONS

Several questions have arisen from peer review of these studies within and outwith my own institution. We hope to address a number of these issues in future studies.

8.2.1 INVESTIGATING THE RELATIVE CONTRIBUTIONS OF SPONTANEOUS AND ENDOGENOUS FIBRINOLYSIS

Plasmin can be generated from plasminogen by t-PA or (single chain [sc]) urokinase (u-PA)[Booth NA, 2006]. Fibrinolysis is usually studied in plasma, where the major focus is the balance between t-PA and it's principle inhibitor, PAI-1. Urokinase activity is hardly ever relevant in plasma, since circulating (sc)u-PA is present at very low concentrations and is rapidly neutralised [Booth NA, 2006]. Thus t-PA has come to be regarded as the key plasminogen activator in the circulation particularly as its level rises dramatically following agonist-mediated release from the endothelium.

Recent studies have emphasised the importance of considering the balance of proteases and inhibitors in fibrinolysis in whole blood, not just plasma, thus allowing assessment of the contribution made by cellular components. A key approach has been the use of model thrombi, formed *in vitro* from whole blood. Formation under flow results in a characteristic thrombus with a cell-rich head and a fibrin-rich tail [Chandler, 1958; Robbie*, et al.*, 1997]. The thrombi, either whole or cut into these two parts, can be sectioned for *in situ* zymography [Mutch*, et al.*, 2002], fixed and stained [Mutch*, et al.*, 2003; Robbie*, et al.*, 1997] or extracted to assess the proteases and inhibitors present [Mutch*, et al.*, 2003; Robbie*, et al.*, 1997]. Fibrinolysis can be measured by incorporating FITC-fibrinogen into the forming thrombi and then following fluorescence release over time [Mutch*, et al.*, 2003; Robbie*, et al.*, 2000].

This is therefore a complex but experimentally tractable system, which has generated several insights into fibrinolytic mechanisms [Mutch*, et al.*, 2007]. During the course of these studies, it was noted that significant spontaneous lysis occurred in the cell-rich head of the model thrombi without the addition of any plasminogen activator. The term "spontaneous" fibrinolysis was adopted to describe it more accurately and to avoid confusion with "endogenous" fibrinolysis.

The effect of spontaneous lysis is sizeable and reproducible. Further work has demonstrated that it results from (sc)u-PA activity and is principally related to platelet and neutrophil content. These findings, and those of others [Gurewich, 2000; Moroz*, et al.*, 1979], question the traditional view that u-PA is involved only outside the circulation, with primary roles in cancer, while t-PA is the main intravascular fibrinolytic enzyme.

In collaboration with colleagues at the University of Aberdeen, the core focus of an ongoing study funded by a BHF project grant is defining the relative efficiency of lysis via (sc)u-PA activation and via t-PA release from the endothelium. Two distinct mechanisms are in play, whereby spontaneous lysis leads to local generation of activity on the surface of cells within the thrombus, while endogenous lysis shifts the balance between t-PA and its main inhibitor, PAI-1, in solution.

We hypothesise that u-PA-dependent and t-PA-dependent fibrinolysis represent temporally and environmentally complementary mechanisms for the dissolution of thrombi *in vivo*. We are using complementary *in vitro* and *ex vivo* models to help

define the precise conditions under which each of the two mechanisms is dominant and investigate their respective potential for further therapeutic development.

8.2.2 DEVELOPMENT OF PROFIBRINOLYTIC DRUGS

Given the importance of the endogenous fibrinolytic system in modulating the outcome of thrombotic events that are central to the pathogenesis of cardiovascular disease, agents capable of shifting the balance in favour of fibrinolysis remain a target for drug development. Such agents may be useful in patients presenting with acute events such as myocardial infarction but may also play a role in the primary or secondary prevention of vascular disease. PAI-1, in particular, is the focus of ongoing interest. Elevated levels are associated with a number of pathological processes that play important roles in the development and outcome of cardiovascular disease including coagulation, fibrinolysis and inflammation. Levels are particularly high in the growing number of patients with metabolic syndrome and it appears that this cohort may benefit most from strategies to inhibit PAI-1 activity.

As part of this body of work, we undertook what we believe to be the first robust evaluation of a small molecule PAI-1 inhibitor, PAI-749, in human blood. Whilst we failed to demonstrate compound efficacy in the *in vitro* or *ex vivo* models we employed, we did observe accelerated *in vitro* fibrinolysis in the presence of a monoclonal antibody against PAI–1. Along with previous encouraging results from preclinical studies, in which a number of inhibiting antibodies and small molecules have shown efficacy, this suggests that the inhibition of PAI–1 remains a potential therapeutic target worthy of further exploration. Data continue to be published [Fjellstrom*, et al.*, 2012] although progress is slow and may relate, in part, to the

physical plasticity of this unusual target. To date, we are not aware of any PAI-1 inhibitor having been tested in patients. We believe that the Badimon Chamber, particularly given its ability to assess the effects of compounds added to the extracorporeal circuit, provides a flexible and robust model with which to help evaluate novel agents able to modulate fibrinolysis for therapeutic benefit.

8.2.3 IS *EX VIVO* THROMBOSIS ENHANCED IN THOSE WITH CARDIOVASCULAR RISK FACTORS OR ESTABLISHED VASCULAR DISEASE?

Ruptured abdominal aortic aneurysms (AAA) are a common cause of death in the UK [Ashton, *et al.*, 2002] with cigarette smoking by far the strongest independent risk factor for development and progression [Blanchard, *et al.*, 2000; Lederle, *et al.*, 1997; Lederle, *et al.*, 2002; Lindholt, *et al.*, 2001]. Although overlap clearly exists between risk factors for atherosclerosis and AAA formation, there is growing evidence to suggest that the underlying mechanisms may be distinct.

Particular interest has focused on the role of thrombus present within the AAA [Kazi, *et al.*, 2003; Touat, *et al.*, 2006]. The presence of laminated intraluminal thrombus is virtually ubiquitous. This thrombus mass is biologically active with fibrinolytic activity demonstrable at the luminal interface [Touat, *et al.*, 2006] and plasma concentrations of components of the fibrinolytic system elevated in peripheral blood [Diehm, *et al.*, 2011; Golledge, *et al.*, 2011; Parry, *et al.*, 2009]. D-dimer, in particular has been identified as one of the potential prognostic biomarkers in patients with AAA [Golledge, *et al.*, 2011; Parry, *et al.*, 2009] and interestingly, plasma markers of fibrin turnover fall following AAA repair [Holmberg, *et al.*, 1999; Yamazumi, *et al.*, 1998]. In addition to its role in the dissolution of fibrin in blood vessels, the

fibrinolytic system is also important in the remodelling of the extracellular matrix, through activation of proteolytic matrix metalloproteinases. These extracellular proteases are involved in AAA expansion and progression towards rupture [Lindholt*, et al.*, 2003; Sakalihasan*, et al.*, 2005]. Thus, the predictive values of fibrinolytic components found in AAA patients mirror the proteolytic activity of the mural thrombus, perhaps explaining the link between the observed thrombus and the immediate risk of rupture [Houard*, et al.*, 2007].

Finally, it has been suggested that intraluminal thrombus in tandem with deranged fibrinolytic function in smokers leads to occlusion of the *vasa vasorum* in aortic wall, resulting in vessel wall ischemia that promotes expansion of the aneurysm.

Taken together, these data suggest that imbalances in the endogenous fibrinolytic system play a major role in AAA expansion although several key questions remain unanswered.

Using forearm venous occlusion plethysmography, we will assess endothelium-dependent vasomotion and acute endogenous fibrinolytic capacity in patients who are current cigarette smokers and have developed AAAs and compare them with age- and sex-matched otherwise healthy cigarette smokers. We will also assess thrombogenicity in these patients using the Badimon Chamber.

We believe that these studies will shed light on some of the pathological processes that drive AAA formation and progression, and particularly address whether there is evidence of impaired fibrinolytic function in patients with AAA.

8.2.4 ARE THE ADVERSE CARDIOVASCULAR EFFECTS OF DIESEL EXHAUST INHALATION RELATED TO THE MAGNITUDE OF THE EXPOSURE?

In previous studies we have exposed subjects to particulate concentrations *circa* 300 $\mu g/m^3$, levels commonly found in heavy traffic, occupational settings, and in the world's largest cities. A significant proportion of this mass is made up of combustion-derived nanoparticles principally generated from traffic. Estimates range from 20% at remote monitoring sites [Lanki*, et al.*, 2006] to 70% in a road tunnel [Geller*, et al.*, 2005]. Exposure to 300 $\mu g/m^3$ for one hour increases an individual's average exposure over a 24-hour period by only 12 $\mu g/m^3$. Changes of this magnitude occur on a daily basis, and are associated with increases in cardiorespiratory mortality [Dockery*, et al.*, 1993]. However, we recognise that many people may not be exposed to this level of air pollution.

Sixteen non-smoking volunteers with normal lung function and no history of respiratory disease will be invited to attend on four separate days at least two weeks apart and receive a double-blind randomised cross-over exposure to filtered clean air or diesel exhaust at 30, 100 and 300 $\mu g/m^3$. Vascular function and thrombotic potential will be determined 6-8 hours after each exposure using venous occlusion plethysmography and the Badimon Chamber.

By exploring the dose-response relationship of these effects we hope to determine safe thresholds of diesel exhaust inhalation with which to inform the ongoing debate surrounding air quality standards.

8.2.5 ARE THE CARDIOVASCULAR EFFECTS OF EXPOSURE TO NEWER 'GREENER' BIODIESEL EXHAUST SIMILAR TO THOSE OBSERVED WITH CONVENTIONAL DIESEL EXHAUST?

The last decade has seen an exhaustive drive towards finding bioeconomical and renewable sources of fuel in order to reduce dependence on fossil fuels. Since 2000 global biofuel production has tripled. Although several potential climatic advantages have been highlighted, there is still much controversy surrounding the ethical and wider environmental repercussions of biofuel production and combustion. Despite huge investment and calls in 2007 by the US Environmental Protection Agency, research into the health effects has been almost completely overlooked; to date there is only one animal study performed that addresses the potential cardiovascular effects of biodiesel exhaust exposure [Brito*, et al.*, 2010]. The study showed that although certain emission parameters were decreased compared with conventional diesel, indices of cardiovascular, pulmonary and systemic inflammation were, in fact, elevated. Studies focussing on the chemical composition of biodiesel exhaust have found that although there seems to be an overall reduction in PAHs, different running cycles and fuel blends may increase levels of toxic and carcinogenic PAH subgroups [Karavalakis*, et al.*, 2010].

Twenty healthy volunteers are currently being enrolled to receive a double-blind randomised cross-over exposure to diesel exhaust generated from standard diesel fuel or exhaust generated from rapeseed oil methyl ester (RME) 30 biodiesel. RME 30 is a mix of 30% RME and 70% standard diesel and has been chosen as it is the most widely commercially available biodiesel in Europe. Several relevant and complementary surrogate markers of cardiovascular health, including *ex vivo* thrombus formation in the Badimon Chamber, will be assessed.

The ongoing debate surrounding the relative merits of biofuel use is fierce and complicated by a number of complex political and ethical considerations. Given the absence of previous human exposure studies, we hope that this work will provide important safety data that should form a crucial part of the debate.

8.2.6 DO ENGINEERED NANOPARTICLES POSE A PROTHROMBOTIC RISK?

Engineered nanoparticles are currently being developed to facilitate the imaging of atherosclerotic plaques and deliver targeted therapies to unstable plaques. However, such particles share many properties with combustion-derived nanoparticles, thought to be principally responsible for the adverse cardiovascular effects of air pollution. To assess the relative risks and benefits of engineered nanoparticles, we need a better understanding of the properties that determine their toxicity, potential for systemic translocation and biological distribution once blood-borne. We have secured substantial funding from the Department of Health (PR-NT-0208-10025) to systematically assess the effects of a range of engineered nanoparticles on platelet activation, platelet-monocyte aggregation and thrombus formation using flow cytometric techniques and by infusion into the extracorporeal circuit of the Badimon Chamber. The role of particle size, surface area, surface chemistry and different imaging moieties will be assessed *in vitro* and *ex vivo* using both in-house synthesised and commercially available nanoparticulate contrast agents. In addition, again using the Badimon Chamber, we plan to assess *ex vivo* thrombus formation in patients with abdominal aortic aneurysms who have received an infusion of a commercially available nanoparticulate contrast agent.

These studies will help to resolve a potentially important paradox in medical imaging and therapeutics: environmental nanoparticulate are toxic to the cardiovascular system even at relatively low concentrations, yet in the quest to enhance cardiovascular imaging the cardiovascular community propose direct intravascular infusion of nanoparticles of unknown toxicity at high concentrations into patients with unstable atheromatous disease. We anticipate that our studies will help define the net clinical benefit of using these agents and lead to the development of safer engineered particles for imaging in patients with stable and unstable atheromatous vascular disease.

8.3 CLINICAL PERSPECTIVE

The initiation, modification and resolution of thrombus associated with unstable atheromatous plaques are central to the pathophysiology of vascular events. The ability to investigate these processes in a clinical model is crucial for the development of novel agents and strategies to treat and prevent a range of cardiovascular disorders.

During this thesis I have demonstrated that the Badimon Chamber is a robust and reproducible model with which to assess *ex vivo* thrombus formation in man. By updating the existing methodology, I have broadened the applicability of the technique. I have demonstrated it has value in assessing novel agents with the potential to modulate exogenous and endogenous fibrinolysis as well as thrombosis. In addition, using it to demonstrate that t-PA released from the endothelium following bradykinin stimulation is functionally active highlights the importance of the endogenous fibrinolytic system and supports further work to investigate its therapeutic value. Whilst the small molecule PAI-1 inhibitor, PAI-749, evaluated here was not efficacious, the Badimon Chamber is well placed to assess other novel compounds and the quest for agents able to enhance fibrinolysis should continue.

Exposure to particulate air pollution is associated with acute cardiovascular events including myocardial infarction. Given that such exposure is virtually ubiquitous and continues to increase in heavily populated developing countries, particulate air pollution is an important risk factor for cardiovascular disease and events on a global basis. Using a well characterised, controlled diesel exhaust exposure system we have demonstrated, for the first time, that exposure to diesel exhaust activates platelets and enhances *ex vivo* thrombosis in healthy volunteers. In addition, for the first time, we

demonstrate that reducing the particulate component using a commercially available particle trap can prevent detrimental effects on several complementary surrogate markers of cardiovascular health. These studies support policy interventions targeting reductions in the traffic-derived component of air pollution including the fitment of particle traps to diesel-powered vehicles in order to limit a range of adverse cardiovascular effects.

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APPENDIX

PUBLICATIONS ARISING FROM THIS THESIS

ORIGINAL ARTICLES

Diesel Exhaust Inhalation Increases Thrombus Formation In Man. Lucking AJ, Lundback M, Mills NM, Barath S, Faratian D, Cassee FR, Donaldson K, Boon NA, Sandstrom T, Blomberg A, Badimon JJ, Newby DE. *Eur Heart J* 2008; **29(24)**: 3043-51

Exposure to Diesel Exhaust Increases Arterial Stiffness in Man. Lundback M, Mills NM, Lucking AJ, Barath S, Newby DE, Sandstrom T, Blomberg A. *Part Fibre Toxicol* 2009;**6**:7.

Effect of the small molecule PAI-1 inhibitor, PAI-749, in clinical models of fibrinolysis. Lucking AJ, Visvanathan A, Philippou H, Fraser S, Grant PJ, Connolly TM, Gardell SJ, Feuerstein GZ, Fox KA, Booth NA, Newby DE. *J Thromb Haemost*. 2010; **8(6)**:1333-9.

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Impaired Vascular Function After Exposure to Diesel Exhaust Generated at Urban Transient Running Conditions. Barath S, Mills, NM, Lundbäck M, Törnqvist H, Lucking AJ, Langrish JP, Söderberg S, Boman C, Westerholm R, Löndahl J, Donaldson K, Mudway I, Sandström T, Newby DE, Blomberg A. *Part Fibre Toxicol*. 2010;**7**:19.

Characterisation and Reproducibility of a Human Ex Vivo Model of Thrombosis. Lucking AJ, Chelliah R, Trotman AD, Connolly TM, Feuerstein GZ, Fox KA, Boon NA, Badimon JJ, Newby DE. *Thromb Res*. 2010;**126(5)**:431-5.

Particle traps prevent adverse vascular and prothrombotic effects of diesel engine exhaust inhalation in men. Lucking AJ, Lundbäck M, Barath SL, Mills NL, Sidhu MK, Langrish JP, Boon NA, Pourazar J, Badimon JJ, Gerlofs-Nijland ME, Cassee FR, Boman C, Donaldson K, Sandstrom T, Newby DE, Blomberg A. *Circulation*. 2011;**123(16)**:1721-8.

Combustion-derived nanoparticulate induces the adverse vascular effects of diesel exhaust inhalation. Mills NL, Miller MR, Lucking AJ, Beveridge J, Flint L, Boere AJ, Fokkens PH, Boon NA, Sandstrom T, Blomberg A, Duffin R, Donaldson K, Hadoke PW, Cassee FR, Newby DE. *Eur Heart J*. 2011;**32(21)**:2660-71.

Endogenous Tissue Plasminogen Activator Enhances Fibrinolysis and Limits Thrombus Formation in a Clinical Model of Thrombosis. Lucking AJ, Gibson KR, Paterson EE, Faratian D, Ludlam CA, Boon NA, Fox KAA, Newby DE. *ATVB* 2013.

BOOK CHAPTER

Pulmonary and Cardiovascular Effects of Nanoparticles in Nanotoxicology: Characterisation, Dosing and Health Effects on Target Organs. Donaldson K, Newby DE, McNee B, Duffin R, Mills NM, Lucking AJ. Chapter entitled Taylor & Francis/Informa Healthcare, edited by N.Monteiro-Riviere and L.Tran